

Molecular Diagnosis in Prenatal Medicine

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Molecular Diagnosis in Prenatal Medicine



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*For Carrie,
Our private prenatal project,
Und für meine Eltern.*

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Introduction:
Molecular Diagnosis and Screening in
Prenatal Medicine.

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Chapter 1

Current screening: age, ultrasound and serum proteins

Cytogenetic disorders are present in about 1 % of live births, with an increased rate for pregnancies in women of advanced age. Avoidance of Down syndrome births is the principal aim of prenatal screening for aneuploidy. The historical indicator of risk was advanced maternal age. Serum screening of placental analytes and ultrasound examination, mainly the measurements of nuchal translucency and nasal bone length during the first and second trimester can be used in conjunction to adjust the age related risk(1-4). The detection rate of all chromosomal defects amounts to over 90 %, but the false positive rate is high with approximately 5 % of the total screenings (5). Consequently, a much greater number of invasive confirmatory tests are performed than actual diagnoses made. A great demand for more accurate and fast methods that can properly determine the risk of a fetus for having a chromosome anomaly exists.

Invasive diagnosis

Prenatal diagnosis of chromosomal anomalies and single gene disorders currently relies on invasive testing with chorionic villus sampling or amniocentesis in pregnancies considered to be at increased risk for these anomalies. As it is an invasive procedure it can cause a miscarriage in up to 1 % of pregnancies.

Invasive testing is advised for pregnancies that bear a high risk of being affected by a chromosomal aberration from family and individual history. Furthermore, women identified as having an increased risk by first trimester screening can opt for genetic testing.

Depending on the gestational age, the invasive procedure can be chorionic villi sampling (CVS) or amniocentesis. Following the sampling a full karyotype is established. This procedure requires an average duration of two weeks for cell culture. Developments in laboratory methods such as Fluorescent In-Situ Hybridization (FISH) and quantitative fluorescent PCR (QF-PCR) of short tandem repeats (STR) (6-8) allow to obtain a first diagnostic answer within 24 – 48 hours. To date, these rapid tests complement but do not replace the full karyotype analysis as they only provide an

answer for a limited number of numerical aberrations (13, 18, 21, X and Y), covering about 70 % of all pertinent aberrations. The diagnosis of major chromosomal abnormalities in this shortened period after the invasive procedure offers the possibility of quick intervention in case of adverse results confirming aberrant ultrasound findings. Additionally, it is discussed whether the rapid tests can fully replace karyotype analysis for pregnancies at a moderate risk for chromosomal aberration, since the tested aberrations amount to about 99.8 % of the disorders in this group (9). An overview of aneuploidy testing methods is presented in chapter 3.

In the context of rapid testing I recently demonstrated that the precision of the TaqMan based multiplex real-time qPCR can be optimized to detect as low as 3:2 differences in template copy number, as is the case for the affected chromosome in fetal aneuploidies. These preliminary results were published in Clinical Chemistry and comprise chapter 4 of the thesis (10). The increased accuracy exceeding the 2:1 difference measurable according to the manufacturers was achieved by implementing special experimental and analytical parameters.

Similar parameters were adopted to enable the determination of rhesus D zygosity using real-time quantitative PCR, chapter 5, which appeared in Swiss Medical Weekly 2003 (11).

We were asked to contribute a book chapter on the method of trisomy detection by real-time PCR for the cutting edge 2nd Edition of “Diagnostic applications of PCR” by Dennis Lo, which appears as chapter 6. The initial investigation was extended by development of an assay for the simultaneous assessment of chromosomes 18 and 21.

We then aimed to prove the diagnostic potential of the concept in a blinded large-scale retrospective study on clinical samples. The results (chapter 7) indicate that real-time quantitative PCR is a powerful tool for the prenatal diagnosis of aneuploidies. The method is straightforward and can produce results of diagnostic value within as little as two hours. With further development of the instrumentation and reagents the test can be easily optimized to a high-throughput method, which would save time and money and would allow to analyze an increased number of targets. Alternatively, the test can be amended to perform testing of the sample directly at the point of care, without the need for technical staff, and results would be available within one hour. The potential speed and simple test set-up of the novel real-time PCR approach are not matched by other rapid methods.

Non-invasive molecular methods for the prenatal diagnosis and screening

As the invasive procedures bear a considerable risk for fetal loss, a long sought goal in prenatal medicine is the establishment of non-invasive tests that allow for prenatal genetic testing. The traditional strategy to accomplish this goal was the isolation of fetal cells found in the maternal circulation. The later discovery of fetal DNA circulating in the maternal plasma has shifted the attention towards this target, which can be found at higher numbers than the scarce cells.

Fetal cells

Fetal cells have been known to cross the placental barrier into the maternal circulation for a long time, but extensive attempts to use them for prenatal diagnosis have not had any consistent success. In a large multi-center trial investigating the approach of recovering fetal cells from the maternal circulation, the NIFTY study (National Institute of Child Health and Development Fetal Cell Isolation Study), various enrichment techniques using fluorescent-activated cell sorting (FACS) or magnetic activated cell sorting (MACS) coupled to FISH analysis have been evaluated for clinical use (15) in our laboratory. This approach has proven to be too labor intensive, with discouragingly low detection and high false positive rates. Although the biologic availability of fetal cells has been demonstrated, the development of practical technology still requires further developments.

Also, attempts at selectively culturing viable fetal cells from maternal peripheral blood have been made. Cell culture could theoretically be used to expand the number of fetal cells for testing. At the beginning of my thesis, a high profile report by Tutschek (12) raised hopes that prenatal diagnosis might be possible by this strategy. We aimed to confirm the reported success of the culture of single clones of fetal progenitor cells from maternal blood. Hemopoietic progenitor cells were cultured from 16 blood samples obtained from women pregnant with a male singleton fetus and single colonies were isolated by micro-manipulation. Examination by multiplex

real-time PCR identified 1'648 as maternal and no colonies of fetal origin were detected. This lead to the conclusion that the clonal expansion of single fetal colonies from the maternal circulation is not feasible by current methods (13). My work, which is presented in chapter 2, as well as other reports, has shown, that the results of Tutschek were not reproducible and findings seem to originate from artefacts in the analysis rather than from cultured fetal cells (comment in Lancet. 2001; Mar 24; 357(9260): 962).

Cell-free circulatory DNA

Until recently, all efforts for the non-invasive recovery of fetal genetic material were aimed at fetal cells. The discovery of significant quantities of circulating cell free DNA by Lo in the plasma of pregnant women opened a new window for diagnosis (14). The free extracellular DNA can be readily extracted from maternal plasma to high purity and quantified by real-time Polymerase Chain Reaction amplification.

It has been suggested that the cell free fetal DNA originates from the placenta (15-18).

Free circulatory DNA: diagnosis of sequences not present in maternal genome

Since it's discovery, the use of free fetal DNA has been limited to the detection of uniquely paternal sequences. The determination of fetal sex by the amplification of Y-chromosome specific sequences has been implemented in the diagnosis of X-linked genetic disorders (19;20). With female fetuses being unaffected, it helps reducing the number of invasive procedures to 50 %. Similarly, the paternal contribution to the rhesus D status in pregnancies of rhesus D negative pregnant women is fairly easy to determine from plasma DNA(21) and is already offered by health services in Holland and Great Britain. The test helps to prevent the prophylactic administering of anti-D immunoglobulins in case of a rhesus D negative fetus (22).

Several studies assess the sensitivity and specificity of fetal DNA detection by amplification of sequences specific to the Y-chromosome and the Rhesus D gene in

the first and second trimester maternal plasma (23). These studies report 95-100 % sensitivity and specificity. Between 1 and 5 % of the total maternal plasma DNA usually correspond to the fetus during the gestational age from 11-17 weeks. Lower amounts of fetal DNA are observed in the first trimester resulting in a reduced detection rate, but fetal DNA from plasma is available in every pregnancy by the second half of the first trimester.

Free circulatory DNA in screening

A current focus in research on cell free fetal DNA investigates its use as a maternal serum marker in early pregnancy for fetal Down Syndrome or other pregnancy related complications. The quantitative measurement of fetal DNA by real-time PCR may complement the major second trimester serum markers. By increasing the specificity of the screening it could help preventing a considerable amount of unnecessary invasive procedures. The median of fetal DNA concentration is reported to be approximately twofold higher in Down Syndrome compared with unaffected pregnancies, but the data is disputed by other reports (24-28). Elevated levels of fetal DNA in the maternal plasma were also reported in pregnancies with preeclampsia and polyhydramnios (29-34). Large sample numbers and meticulously accurate, standardized quantification are demanded to allow final conclusions.

An impediment in the generation of accurate data is the low number of fetal sequences in the plasma, such that the samples quantified by the real-time PCR have copy numbers close to the detection limit, and the quantitative value of the results is reduced by increased variability of the method and sampling effects. As we could observe in a recent comparison study between five laboratories, even use of the identical sample material and implementation of the same DNA extraction and real-time quantitative PCR procedures can result in major differences of the finalized data. In the comparison of numerical values from different studies using different protocols, major discrepancies can be expected (35). In the context of clinical application it will be important to standardize several procedural steps which otherwise can lead to variability of the quantitative data. Also, a more precise description of the quantification procedures needs to be included when reporting results.

In chapter 10 we present an approach to address one of the major hindrances in the generation of precise and relevant data, the scarce nature of the material to be quantified. We amended an existing but sub-optimal real-time quantitative PCR protocol specific to the multicopy locus DYS14 on the Y-chromosome and evaluated its performance in comparison to the currently used SRY targeting protocol. Our evaluation shows that the new assay performs with an increased precision and sensitivity. As most reports quantify samples surprisingly close to the theoretical detection limit, we tried to highlight several important points to generate accurate data. Furthermore we show that the assay can be used for sex determination by applying a cut-off to exclude false positive results.

Most studies generate data from pregnancies with male fetuses, as sequences on the Y chromosome are unique DNA markers absent from the maternal genome. This approach is only applicable to the male 50 % of the pregnancies, but straightforward to perform. In order to use the fetal DNA as a screening marker, a gender-independent fetal DNA marker that can be assayed by real-time PCR is needed. Polymorphic sequences are presently used in the clinical samples that are investigated for fetal rhesus status to ascertain the presence of adequate amounts of fetal DNA (36). Although a number of sequences has to be examined in each pregnancy to ascertain distinction between fetal and maternal, this approach is still relatively facile to implement.

Mutation detection with free DNA

A caveat of the approach is that the DNA present in the circulation is predominantly of maternal origin and interferes with molecular analysis of the fetal DNA. Hence, only paternally inherited fetal loci that are clearly distinct from maternal genomic sequences can be readily examined. Paternally inherited mutant genes in compound heterozygous genetic disorders may also be detected if the paternal mutation is dissimilar from the maternal allele, as for example a four base deletion causing thalassemia (37). In general, the detection of fetal single gene disorders is at least cumbersome and often impossible with current methods. Likewise does the

determination of gross chromosomal abnormalities require another advancement in the technology before it is feasible in a non-invasive manner.

I participated in the successful enrichment of fetal DNA as compared to the maternal fraction from plasma by agarose gel electrophoresis mediated size separation. By this approach, which is presented in chapter 8 (38), we were able for the first time to detect fetal STR-alleles in size-separated DNA isolated from maternal plasma. Very recently it has also been shown by Lo that the majority of the fetal circulatory DNA in the plasma of pregnant women is of short length, which is contrary to the much longer total circulatory DNA, confirming the validity of our approach (39).

We applied the strategy of enriching the proportion of fetal DNA for the detection of point mutations in the beta-Globin gene causing thalassemia. Our successful analysis of 15 of 16 samples is presented in chapter 9.

A recent report (40) claimed the recovery of increased proportions of fetal DNA from maternal plasma, rendering DNA extracts of in average 25 % fetal origin. The decreased levels of maternal DNA were claimed to be due to stabilization of cells by formaldehyde at the time of blood sampling. However, when we tried to repeat the results, our quantification by real-time PCR could not confirm this massive increase of the fetal percentage (chapter 11).

Circulatory RNA: discovery, stability, promises

The presence of cell free fetal DNA in maternal plasma has revealed significant clinical potential for the prenatal diagnosis of fetal genetic diseases and pregnancy-associated complications. Extending beyond plasma DNA, a new field of investigation has opened by the demonstration of the cell free form of fetal RNA in maternal plasma, which is surprisingly stable and holds promise for non-invasive profiling of placental gene expression (41). As gender independent fetal DNA markers are laborious to implement, efforts to identify screening markers for pregnancy-associated disorders, such as preeclampsia or aneuploidy, now focus at identifying placentally expressed mRNA. To date only a few reports present quantitative data on this phenomenon. They indicate that mRNA from maternal plasma may be used as a gender independent marker group that provides a suitable

screening tool for pregnancy associated pathologies. Quantitative analysis of mRNA has proven to be very complicated in other fields of research, with great variability of the data due to the instability of the target molecules and variable efficiencies of the reverse transcription step. Also, plasma markers of fetal origin and clinical relevance await to be established.

In summary, the present findings point mainly to the possible use of fetal DNA or RNA as additional screening analytes for chromosomal anomalies and other complications of pregnancy. The ultimate goal is to develop non invasive prenatal procedures that can be used for the diagnosis of chromosome and single gene disorders.

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Inability to Clonally Expand Fetal Progenitors from Maternal Blood.

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Chapter 2

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Abstract

Objectives: To confirm the recent report of the culture of single clones of fetal progenitor cells from maternal blood.

Methods: Hemopoietic progenitor cells were cultured from 16 blood samples obtained from women pregnant with a male singleton fetus. Single colonies were isolated by micro-manipulation and examined by multiplex real-time PCR.

Results: Of a total of 1,674 colonies examined, 1,648 were identified as being maternal. No colonies were detected of fetal origin.

Conclusions: The clonal expansion of single fetal colonies from the maternal circulation is not feasible by current methods.

Key Words

Fetal cells, Maternal blood culture, Polymerase chain reaction

Introduction

The isolation of fetal cells, specifically fetal erythroblasts, from the blood of pregnant women offers a unique opportunity for the non-invasive risk-free examination of fetal genetic traits [1, 2]. Previous proof of concept studies have shown that both fetal aneuploidies as well as inherited single gene disorders can be detected by the analysis of such enriched fetal erythroblasts by either FISH (fluorescence in situ hybridisation) or PCR (polymerase chain reaction) [1, 2]. These pioneering studies have also indicated that the frequency of fetal cells in the maternal periphery is very low, being of the order of 1 in 10^6 to 1 in 10^7 maternal nucleated cells [3]. Due to their scarcity, only very few fetal cells are recovered from normal maternal blood samples, which implies that a diagnosis may be based on the analysis of one or two cells. Our own experience in the simple determination of fetal sex by the use of FISH for the X and Y chromosomes on enriched fetal erythroblasts has shown that optimal specificity is attained when 3 or more cells can be evaluated [4]. In a similar manner, it has been shown that the examination of single cells by PCR is also prone to several technical problems of which the most evident is allele dropout, a PCR phenomenon whereby only 1 allele of a particular locus is efficiently amplified [5, 6]. In this manner heterozygous loci could be incorrectly determined as either being homozygous wildtype or of greater concern as homozygous affected mutant [5]. An approach we have developed to counter this problem is the independent analysis of several candidate fetal cells by single cell PCR, by which means we have shown that diagnostic efficacies of almost 100% can be attained from the analysis of 4–5 fetal cells despite an allele dropout rate of over 40% [7]. The caveat of this approach is that it requires the reliable detection of at least 5 fetal cells, a feature we have not always been able to achieve, even when using a highly optimised enrichment protocol with normal maternal blood samples [8]. A further disadvantage of only being able to examine a few differentiated non-dividing cells is that it is impossible to obtain conclusive information regarding the entire fetal karyotype. For this reason, several researchers have attempted to culture fetal hemopoietic progenitor cells which have been enriched from the maternal circulation. Reports indicating that such approaches may be feasible have been made by Valerio et al. [9, 10] as well as by the group of Di Renzo [11]. In their studies these researchers have used either anti-CD34 or erythropoietin receptor based enrichment procedures in order to obtain sufficient fetal

progenitors which could then be amplified in culture. By these means Valerio et al. [12] have reported upon the successful identification of fetal aneuploidies. As these enrichment procedures and culture conditions are quite complex they are deemed as being too difficult to adapt to a routine clinical setting. In this regard, the recent report by Tutschek et al. [13] of the clonal expansion of fetal progenitors directly from non-enriched maternal blood samples has considerably raised hopes that it may be possible to obtain sufficient fetal cells for complex genetic analyses by less technically challenging means. The aim of our experiments has been to confirm this observation in an independent manner.

Materials and Methods

Blood Samples

For this study we recruited 16 women with normal pregnancies between 13 and 41 weeks of gestation who had been identified by routine ultrasound examination to be carrying a singleton male fetus. None of these women had an invasive prenatal diagnostic sample performed in this pregnancy prior to blood letting. The Institutional Review Board of the University of Basel granted approval for this study. Written informed consent was obtained in all instances.

Isolation of Peripheral Blood Mononuclear Cells (PBMC)

Isolation and culture conditions were used as described by Tutschek et al. [13]. In brief, 7-ml maternal blood samples were collected into a vacuum tube containing EDTA as an anticoagulant (Sarsted, Nümbrecht, Germany). The samples were all processed within 6 h. The mononuclear cells were separated by single-density gradient centrifugation (Ficoll-Paque, Amersham, Uppsala, Sweden), washed twice in phosphate-buffered saline (PBS) and resuspended at a density of 10^6 cells/ml in PBS.

Semisolid Culture of Erythroid Progenitors

The cells were cultured in 24-well plates, 0.8 ml per well, at densities of 0.15 million cells/ml, in a semisolid 1% methylcellulose medium for the assay of human clonogenic hematopoietic progenitors, containing 30% FBS, 1% BSA, 10^{-4} M 2-mercaptoethanol, 2mM L-glutamine, 50 ng/ml rh SCF, 20 ng/ml rh GM-CSF, 20 ng/ml rh IL-3, 20 ng/ml rh IL-6, 20 ng/ml rh G-CSF and 3 U/ml rh epo in Iscove's modified Dulbecco's medium (Methocult TM GF+ 4435, Stem Cell Technologies).

Micromanipulation and Lysis

After 10 days of cultivation, single colonies containing 150 cells were isolated by micro-manipulation: 100- μ l glass micropipettes were drawn into fine micro-capillaries using a Leitz needle puller (Leica, Basel, Switzerland). Single colonies were drawn into these micro-capillaries under microscope guidance. Single colonies were transferred into a single PCR reaction vessel and lysed at 50°C for 1 h in 17 μ M SDS containing 400 ng/ μ l proteinase K.

TaqMan Real-Time PCR

The lysates were analysed in duplicates by the TaqMan real-time multiplex PCR, amplifying the SRY (sex determining region) locus on the Y-chromosome to identify male fetal clones, and the ubiquitous GAPDH (glyceraldehyde 3-phosphodehydrogenase) gene on chromosome 12 as a positive internal control as described previously [14].

Results

In this study, we examined 16 maternal blood samples from women pregnant with a singleton male fetus as identified by ultrasonography. Following culture under conditions optimised for the expansion of erythropoietic progenitor cells in semi-solid medium, single colonies were isolated by micro-manipulation. These were then examined by a real-time multiplex PCR assay, which permits the simultaneous detection of the male Y chromosome and the ubiquitous GAPDH gene. In this manner, it would be very easy to distinguish between cells of male fetal origin and those which are of maternal origin, as the former would be positive for both the Y chromosome and the GAPDH gene, whereas the latter would only be positive for the GAPDH gene. In our experiment we isolated a total of 1,674 individual colonies by micro-manipulation, varying from 50 cells to several thousand cells per colony. As these clones had been grown in semi-solid medium, they are completely clonal in origin having originated from a single cell. In our real-time PCR analysis of these single colonies we obtained efficient amplification in 1,648 clones (98.4%) (table 1). Only in 26 of the isolated colonies could we not obtain any reliable amplification (1.6%). All of the 1,648 tested colonies proved to be positive solely for the GAPDH control gene, but not for the male specific SRY locus. They were determined to be of maternal origin. We could detect one colonies which was clearly male and of fetal origin.

Culture ID	Gestational age, weeks	Colonies examined	Colonies GAPDH+/SRY-	Colonies GAPDH+/SRY+
A	13	111	111	0
B	14	129	129	0
C	20	100	100	0
D	20	116	115	0
E	26	80	80	0
F	28	64	63	0
G	29	74	74	0
H	29	65	65	0
I	31	95	94	0
J	35	127	121	0
K	35	98	98	0
L	35	127	116	0
M	36	192	191	0
N	37	132	132	0
O	40	45	43	0
P	41	119	116	0
Total = 16	n.a.	1,674	1,648	0

Table 1. Examination of single culture colonies by multiplex TaqMan PCR

Discussion

In this study we have attempted to confirm a recent report [13] regarding the very simple clonal expansion of fetal progenitor cells from maternal blood without the need for a specific enrichment step. In order to identify colonies of fetal origin, Tutschek and colleagues used a complex PCR assay for highly polymorphic microsatellite markers, also termed short tandem repeats (STRs). As this assay is quite tedious to perform, we have elected to use a simpler and more rapid method, namely real-time multiplex PCR for the male SRY locus and the ubiquitous GAPDH gene [14, 15]. Since we were only examining pregnancies with singleton male fetuses, any colonies of fetal origin would be positive for both the SRY locus and GAPDH gene. On the other hand, colonies derived from the mother would only be GAPDH positive. The advantages of this assay over the STR one is that it has a much higher throughput in that up to 96 different PCR reactions can be examined simultaneously. The TaqMan real time PCR system is also less prone to contamination as the PCR reaction is monitored directly in the reaction vessel on-line. There is, hence, no need to open the PCR reaction vessels in order to examine the PCR product. It is at this step that the contamination of subsequent reactions by the presence of highly amplified target DNA sequences is most likely to occur. Furthermore, since the assay is quantitative, purely fetal colonies will be expected to have the correct 1:2 gene dosage ratio of the SRY locus located on the single Y chromosome to the autosomal GAPDH gene located on chromosome 12. In this manner, we should be able to detect any contamination with PCR products or extraneous male DNA. In our study, which attempted to duplicate the report of Tutschek et al. [13] as closely as possible, we were unable to confirm the presence of any clonal colonies of fetal origin in maternal blood cultures. Since we have considerable experience with the culture of early fetal hemopoietic progenitor cells [16], it is unlikely that our lack of success is due to a technical deficit. Rather, our result may reflect upon some discrepancies in the initial publication which have recently been commented upon by the group of Fisk in London [17]. The chief criticism raised was that Tutschek and colleagues reported presence of colonies of mixed origin, a strange feature as colonies which are cultured in semisolid media arise from a single progenitor cell and are therefore truly clonal in origin. In our view, it appears that Tutschek and colleagues may have been misled by an error in the complex STR PCR assay they were using. Due to their highly

repetitive nature, usually consisting of a varying number of CG repeats, it frequently occurs that the PCR polymerase incorrectly amplifies the correct length of the STR marker under investigation [5, 7]. Under such conditions it is easy to misinterpret the incorrectly amplified allele and consequently regard it as being indicative of fetal rather than of maternal origin. An example of such an artefact is illustrated in figure 1, where two different microsatellite patterns are obtained from the same DNA source solely due to incorrect amplification of the polymorphic marker interrogated. Such incorrect amplifications frequently occur when dealing with small cell numbers [5, 7], as would be the case when dealing with single micro-manipulated colonies. Of further note is that Tutschek et al. [13] did not report the detection of any male colonies despite the use of a sex determining amelogenin specific PCR reaction. Therefore, our data suggest that the culture of fetal progenitor cells from the maternal circulation is not feasible by the described method, and that it would probably be best to involve some enrichment step, such as anti- CD34 [9–11], in order to obtain a higher proportion of fetal hemopoietic precursor cells.

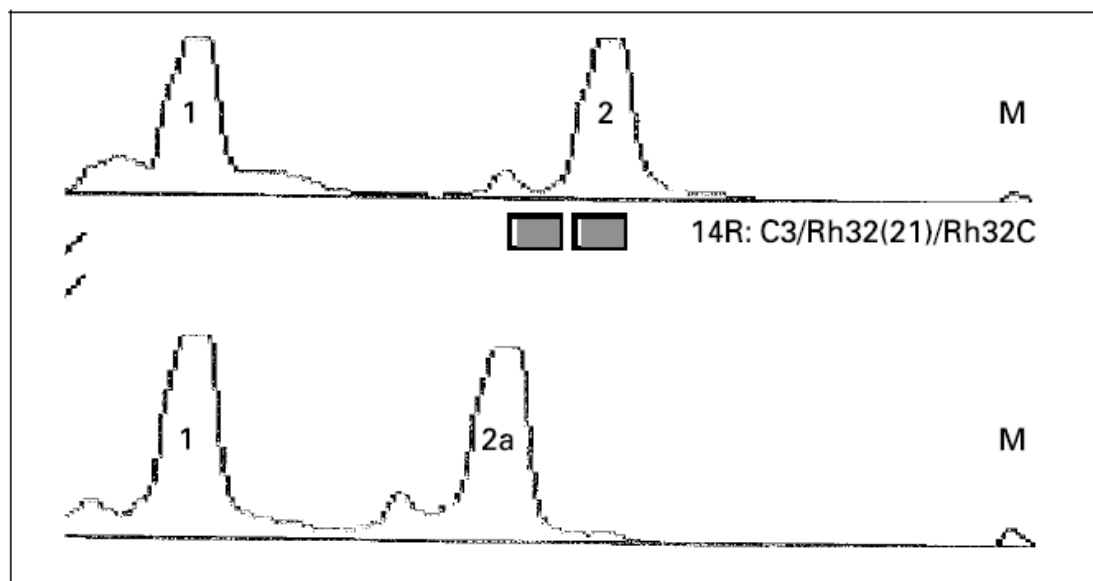


Fig. 1. PCR analysis of highly polymorphic microsatellite markers. In this PCR analysis of a highly polymorphic microsatellite marker two differently sized amplicons are obtained due to incorrect amplification of the allele investigated. Top: Alleles 1 and 2 are correctly amplified. Bottom: Allele 1 is amplified correctly, whereas allele 2 is incorrectly amplified and thereby yields a fragment of incorrect size (2a). This can easily be misinterpreted as being a different allele, e.g. of paternal rather than maternal origin.

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PCR Methods for the Detection of Fetal Aneuploidy and Unbalanced Chromosomal Rearrangements.

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Chapter 3

Introduction

In every pregnancy there is a risk that the fetus may have aneuploidy. Until about two decades ago this risk used to be assessed based on maternal age and family history only. Since then other factors, which affect this risk have been discovered and some of them have been introduced into clinical practice of genetic counseling. Currently the parameters that are employed for prenatal screening in the first trimester are: nuchal translucency (NT) measured by ultrasound (NT is on average increased in fetuses with trisomy 21), nasal bones presence assessed by ultrasound (nasal bones are absent in the majority of trisomy 21 first trimester fetuses), Pregnancy Associated Plasma Protein A (PAPP-A in maternal serum is on average reduced in trisomy 21 fetuses), and free beta subunit of human chorionic gonadotrophin (free beta hCG in maternal serum is on average increased in trisomy 21 fetuses) measured in maternal serum. Deviations of the examined parameters from the median for the normal population are converted into a risk factor by which the a priori risk is multiplied so as to arrive at the final risk of fetal disorder. If the risk of fetal aneuploidy is high enough to justify an invasive test (usually the cut-off value for the risk of birth of a child with trisomy 21 is around 1 in 300 – equivalent to the risk in a 36 year old woman at birth), amniocentesis or chorionic villi sampling is advised.¹

Screening utilizing the above mentioned parameters in the first trimester to derive the risk is the most efficient way to screen for trisomy 21, trisomy 18 and 13, triploidy and monosomy X. This method of screening enables fairly good discrimination between healthy and aneuploid fetuses: in a general (low risk population) detection rates of the aforementioned aneuploidies (without nasal bones assessment) are close to 90% with a false positives rate of 5%. Recent evidence shows that the addition of nasal bones assessment would either boost the sensitivity of trisomy 21 screening to around 97% at 5% false positives or at the sensitivity of 90% decrease the false positives rate to around 1%.²

Screening is rational only when a large proportion of population is tested, which means that even with ideal implementation of the test, a large number of women will present with increased risk of the fetal disorder. This calls for a cytogenetic test, however, this is expensive and takes long time to obtain the result, which is unacceptable for many women. The most common procedures to obtain fetal tissues for cytogenetic testing are amniocentesis, chorionic villus sampling and fetal blood sampling. These procedures are connected to a small, but definite risk of fetal death.

Conventional cytogenetics using Giemsa banding of metaphase chromosomes has proven to detect a wide range of abnormalities with high trustworthiness, deserving the position of a gold standard in prenatal diagnosis. Because this technology is so reliable, it has not changed much for the last 30 years, even earning for this reason an affectionate name of “the alive fossil”. This conservatism in the era of rapidly evolving molecular methods is however not entirely kept, as cytogenetics has in recent years, combined with molecular biology, gone through renaissance with resulting development of FISH and CGH with all their varieties. The unique and unquestionable merit of classical cytogenetics is the ability to screen the whole genome in a “single test” and to detect balanced rearrangements. The resolution of classical cytogenetics is of the order of only 4 to 6 million base pairs, which constitutes one of the most serious drawbacks. Classical cytogenetics is subject to technical problems such as culture failure, external contamination and selective growth of maternal cells. Quantitative interpretation of banded karyotypes can be limited by cell-to-cell variability in chromosome condensation and staining characteristics. The subjective nature of the banded karyotype analysis also potentially complicates interlaboratory comparisons of the size or staining characteristics of specific lesions or polymorphisms.³ Among other earnest problems are high cost, low throughput, long turnaround time, limitations of suitable test material to viable, dividing cells and requirement for unique, highly qualified staff.

Since balanced chromosomal rearrangements are usually associated with a normal phenotype, in prenatal diagnosis detection of unbalanced rearrangements and particularly autosomal trisomies is principal.

Search for a molecular biology method that would replace or at least augment classical cytogenetics is underway because it is expected that DNA-based methods will be cheaper, faster and will have greater resolution. The time to result is a particularly important issue, since more and more women are referred for invasive prenatal diagnosis with very high risk after prenatal screening and the long time necessary for the classical cytogenetics is unacceptable for them.^{4,5,6}

There are many molecular techniques that potentially may be used to detect the underlying chromosome copy number changes in unbalanced rearrangements: fluorescent in situ hybridization (FISH), Southern blots, loss of heterozygosity (LOH) assays, microarray technology, comparative genomic hybridization, automated nuclear DNA cytometry, different modifications of quantitative polymerase chain reaction (Q-PCR). Nevertheless some of them are not practical in routine prenatal

diagnostic setting for different reasons: requirement for a large amount of DNA (Southern blot, LOH), high cost and labor-intensity (FISH, CGH, microarrays), inability to run in multiplex assay (FISH, LOH).

In this article we will focus mainly on the PCR-based molecular methods, that may be expected to come into practice as they are amenable to automation and high throughput.

Southern blotting

In Southern blotting, DNA fragments separated by gel electrophoresis are transferred to the support membrane and the results can be analyzed spatially in terms of molecular weight or relative position. In quantitative Southern blotting copy number changes of specific DNA sequences are assessed by measuring the intensity of the bands by means of densitometry. Deletions or duplications can also be detected if they delete or duplicate a restriction site. Special equipment (e.g. phosphorimager), providing linear dose-response curve is necessary for quantitative measurements since classical photography provides non-linear curve. Quantitative blotting is a relative quantitation method and, therefore, relies on the inclusion of one or more internal controls or reference sequences; quantitation of DNA is relative to this reference sequence of known copy number. However quantitative inference can be undermined by several factors, e.g. densitometric measurement utilizing ethidium bromide can be complicated by irregular backgrounds and diffused bands⁷.

Quantitative Southern blotting requires large amounts of DNA, therefore it is not suitable for prenatal diagnosis. The process is also cumbersome and requires strictly controlled conditions.

Fluorescent in situ hybridization

Fluorescent in situ hybridization (FISH) utilizes fluorescent-labeled probes, which are fluorescently stained single-stranded DNA segments complementary to regions of

individual chromosomes. These probes hybridize with target DNA sequences in the sample and can be identified using fluorescence microscopy. The light signals emitted by the fluorescent stain enable enumeration and spatial analysis of complementary sequences and identification of chromosomal abnormalities, such as trisomies, monosomies, and duplications.⁸

DNA probes used in prenatal diagnosis of aneuploidy by FISH can be categorized in the following way:

- probes consisting of tandemly repeated human DNA sequences, such as centromeric alpha satellite sequences, which are often used for identification and enumeration of specific chromosomes in interphase nuclei.
- Unique sequence probes, which are used for identification of small regions of the genome which may be deleted or duplicated in certain syndromes and are often missed by conventional cytogenetics (e.g. Williams and DiGeorge syndromes).
- Mixtures of unique probes from the same chromosome or chromosome segment which are used to stain, or "paint," large sections of the entire chromosome. Such staining allows analysis of the chromosome number as well as identification of additions and translocations in metaphase cells.⁹

Fluorescent in situ hybridization (FISH) studies of interphase cells avoids the need to culture cells and reduce the time required for the diagnosis. However, this technique is relatively labor intensive, requires intact cells and technical expertise, and is unable to detect balanced rearrangements and imbalanced aberrations involving chromosomal segments other than the sequences complementary to the probe set used. The major aneuploidies diagnosed prenatally by most often used probes set involve the autosomes 13, 18, 21, and sex chromosomes. FISH on interphase nuclei provides an initial rapid screen preceding the full cytogenetic evaluation.¹⁰

PCR

PCR is the most widely used technique of genetic analysis, owing to the combination of the highly specific hybridization and exponential amplification of the target sequences. In quantitative PCR, the amount of PCR product is proportional to the quantity of the initial target sequence, as long as the reaction is stopped during the exponential phase. The product's starting amount has to be calculated from quantities of the exponential phase as end point measurements are not precise enough.

Of the molecular methods amenable to quantitative study the polymerase chain reaction has several advantages: it is less labor intensive and more sensitive than in situ hybridization and, unlike Southern blot analysis, does not require transfer to a membrane and hybridization for quantitation.

When using PCR for quantitative measurements, the following issues need to be kept in mind:

- the measurement should be made during the reproducible exponential phase of the amplification. Consequently, the number of cycles may not be exceedingly high, as with very high cycle numbers the competing products and other factors decrease amplification efficiency, causing plateau effect.
- the concentration of the starting DNA may not be very low, as with very low sequence copy numbers the early phase of amplification is stochastic in nature, (e.g. allele drop-out phenomenon)
- In multiplex PCR presence of multiple primer pairs compromises the robustness of the reaction and reliability of quantitation.¹¹
- Comparisons “between wells” may not be valid, because of small deviations resulting e.g. from pipetting errors, alterations in polymerase activities etc. (This shortcoming would not be overcome even by post-run data analysis^{12,13}.)

Performing a quantitative PCR reaction to determine ploidy of the sample, an internal standard must be implemented, which enables relative quantitation. While diagnosing aneuploidy of a given chromosome, sequences specific for other autosomes may be chosen as such standard. The detection of fetal aneuploidy is a particularly difficult task, involving more precision than usual quantitative applications, because it requires unequivocal discrimination of quantities of the DNA being in the ratio of 3:2. Most of the described methods enable distinguishing between the quantities of the order of at most 2:1.

The qPCR methods suitable for the detection of fetal aneuploidy may be divided in two groups: assays in which measurement is performed by measuring the amount of

products after additional analytical steps and assays in which measurement is performed during the very process of amplification. Quantitative fluorescent PCR, Multiplex Amplifiable Probe Hybridization (MLPH), Multiplex Probe Ligation Assay (MLPA) and melting curve of SNP after the PCR belong to the former and real-time quantitative PCR to the latter group.

Quantitative Fluorescent PCR

Historically the first attempt for PCR-based detection of aneuploidy involved PCR amplification of small-tandem-repeat (STR) markers located on a given chromosome, and analysis by fluorescence-based methods to identify the presence of an additional allele on the third copy of the chromosome or absence of the second allele, denoting its monosomy. This DNA approach was first used for the diagnosis of monosomy X, and has since been modified to include the identification of some other common trisomies. Many studies have assessed the clinical usefulness of this technique.^{14, 15, 16} The main advantages of this approach for the prenatal diagnosis of trisomy 21 Down's syndrome, on DNA extracted from uncultured samples of amniotic fluid, are the small amount of fluid required and the speed and accuracy with which the test can be done. The technique can be automated and 96 samples processed simultaneously, and results available within the same day. It is also objective, but requires special software for interpretation and the development of a set of markers designed particularly for this purpose.¹⁷ After amplification of chosen polymorphic sequences for a limited cycle number, the fluorescent labeled PCR products are analyzed by electrophoresis. Quantitative assessment of copy number can be performed by measuring the area under the curve of the specific peak on the electrophoregram. A normal result shows two different-sized alleles with peaks of equal intensity on electrophoregram. A trisomic sample shows either three different-sized alleles of equal intensity or two peaks with a ratio of 2/1. A single peak is equivocal: it could indicate the presence of either two or three chromosomes carrying the same size allele; thus a normal sample cannot be distinguished from that of a Down's syndrome fetus with this marker and an analysis using a different, informative allele is necessary.

Up to now, the STR-based strategy has been the method of choice for molecular detection of aneuploidies. Although attractive, this method has several disadvantages.

At least two highly heterozygous short tandem repeat (STR) markers are needed to determine trisomy 21 using QF-PCR with fluorescent STRs.¹⁸ In some cases, 3, 4 or more markers are used, increasing the complexity of the PCR.¹⁹ Levett et al., in a large prospective study of 5000 amniotic fluids, were not able to conclude for 1 chromosome in 2% of the samples because 7 out of 8 markers showed a homozygous peak.²⁰ The major drawback here is the need for an informative polymorphism, as even when using multiple sequences, in a proportion of patients no informative sequences may be found. The polymorphic loci show different frequencies in different populations, as a result polymorphisms found to be optimal in one population may be ineffective in another population.²¹ A further Drawback of the assay is that polymerase stuttering can lead to products of false length which may lead to misdiagnosis. Certain polymorphisms are more prone to this error and usually replaced by better ones. Such approach can detect aneuploidy resulting from first or second meiotic divisions. This is a semi-quantitative test, with less need for precision than pure quantitative measurement. Summing up, to detect trisomy using polymorphic sequences one needs either 3 alleles of different length with equal amounts of products or a 2-allele pattern characterized by a 2:1 ratio.

Maternal cell contamination is associated with additional peaks and distortion of the ratios of the QF-PCR peaks even if the maternal cells are present in low number. However, admixture of maternal cells can be confused with mosaicism irrespective of the method and constitutes a problem for all, not only PCR based techniques.²²

Multiplex ligation-dependent probe amplification (MLPA)

Multiplex Amplifiable Probe Hybridization (MAPH)

MLPA is a recently developed PCR-based quantitation technique, that enables robust quantitation of up to 40 sequences in a single test, using only one pair of primers. By using identical primers, the reaction efficiencies are equal for each target. In this approach, not sample DNA but probes added to the samples are PCR amplified and their amplification products quantitated. The template amplified in this reaction consist of two oligonucleotide probes, which after specific hybridization to a target sequence undergo ligation. One of these oligonucleotides has a common sequence used as a primer for the PCR reaction at 5' end and a specific sequence, hybridizing to

the target sequence at 3' end. The other oligonucleotide consists of three parts: At the 5' end a sequence, that hybridizes to the target sequence adjacent to the sequence recognized by the former probe, a common sequence used for amplification at the 3' end and a non-hybridizing so called "stuffer" sequence of variable length in between. During the reaction each oligonucleotide probe of a pair hybridizes to its target sequence adjacent to the target sequence of the second oligonucleotide and subsequently the two parts of the probe are ligated by a ligase, which is very sensitive to probe-target mismatches close to the ligation site. Then the DNA is melted, ligase inactivated and the PCR performed. The mismatch at, or very close to, ligation site or inability of one of oligonucleotides from the pair to hybridize prevents subsequent amplification. The lengths of the stuffer sequences and target sequences are chosen in such a way, that the resulting PCR products differ in size (by around 6 to 9 bp), permitting to distinguish the products originating from different sequences by their length. The PCR products subsequently undergo capillary electrophoresis and the fluorescent peaks are used for quantification. A relative probe signal is calculated for every probe (length) by dividing each individual peak area by the sum of the peak areas of all the probes in the sample and expressed as a multiple of the relative probe signal obtained in a control sample. The expected value for euploid sample is close to 1 and for trisomic sample is close to 1,5. The first blind, prospective clinical trial found this new method robust and reliable. New trials are underway.²³

MAPH is another recently developed PCR-based quantitation technique enabling quantitation of multiple DNA sequences. In MAPH sample DNA is denatured and bound to a nylon membrane. In this technique, like in MLPA, not the sample DNA, but the probes, hybridizing to this DNA are amplified. The so called amplifiable probes used in this method are between 100 and 600 bp long. Their sequences correspond to the different loci tested and are flanked by the same primer-binding sequence. Amplifiable probes, matching the loci which are to be quantitated are hybridized to the immobilized DNA. Because the probes are in surplus, every sequence to be recognized in the sample DNA is occupied, so there is a direct relation between the copy number of the target sequences in the sample DNA and amount of the bound probe. The filter with bound sample DNA and hybridized probes is thoroughly washed, to remove any unbound probes. Then the filter is incubated at 95°C to release the probes, which are subsequently amplified for around 20-25 cycles. The amplification is stopped during the exponential phase of PCR. One of the primers

is usually coupled to a fluorophore, to enable detection and measurement of band intensity after gel or capillary electrophoresis. To our knowledge MAPH has not been used yet to screen for aneuploidy, nevertheless potentially it is a method that in a single run could quantitate multiple (around 40) sequences from the genome, for example detecting common aneuploidies and microdeletion syndromes.

Both MLPA and MAPH rely on sequence-specific probe hybridization to genomic DNA, followed by amplification of the hybridized probe, and semi-quantitative analysis of the resulting PCR products. The relative peak heights or band intensities from each target indicate their initial concentration. The two techniques differ in the ease with which probes can be generated in house, and the labor intensity of performing the assay.²⁴

Allele Quantification Combined with Melting Curves Analysis of Single-Nucleotide Polymorphism Loci

In this technique PCR products amplified from the two different alleles of a pair of heterozygous chromosomes are distinguished by temperature-dependent loss of fluorescence by hybridization probes which is converted into two "melting peaks" centered on the melting temperature specific for each allele. In euploid DNA, for each heterozygous SNP, an allele ratio of 1:1 is anticipated, whereas in trisomic samples ratios of 1:2 are expected. The representation of each allele is estimated by the area under the curve of the derivative melting curve in a post-PCR melting step.²⁵ The measurement can be performed in the same well and in a real-time PCR instrument, avoiding additional sample handling or processing.

The disadvantage here is need for an informative polymorphism, as even when using multiple sequences, in a proportion of patients no informative sequences may be found.. In theory, a panel of six independent SNPs, each heterozygous in 50% of the population, provides an accuracy >98% if diagnosis is established with only one heterozygous locus. In a clinical test that would require confirmatory data from at least two SNPs, informative data would be obtained for more than 89% of the population.

The assay is rapid, with little sample demand, but requires several control reactions due to variability between tests.

Real-time quantitative PCR (qPCR):

One of the most robust and versatile tools for the measurement of the copy number of nucleic acid sequences that has recently been developed is real-time quantitative PCR. It gained popularity because it is more sensitive, specific and reproducible than previous techniques and owing to these features it has been employed for the analysis of gene expression, determination of gene deletions or duplications in various areas of research. It is worth to point out that recent instruments make it possible to obtain results of certain tests after 15 minutes. In comparison to conventional PCR, real-time PCR also offers a much wider dynamic range of up to 10^7 -fold (compared to 1000-fold in conventional qPCR). This means that a wide range of ratios of target and normaliser can be assayed with equal sensitivity and specificity.

This technique requires a thermocycler combined with an optical device for excitation of fluorochromes and detection of emitted photons. Real Time qPCR, unlike “classical” PCR, enables monitoring of the accumulation of the product during the amplification process, which allows abandoning the gel separation phase. Consequently, the analysis is performed in a single well in a closed system without further sample handling. Accumulation of the product can be detected by several different fluorescence markers: intercalating dyes, molecular beacons, dual-labelled probes. Of these, intercalating dyes are the most prone to false results, because of the appearance of the non-specific double-stranded products. The TaqMan real-time qPCR is based upon fluorescence produced by the 5' nuclease method: A so called TaqMan dual labelled probe with a fluorescent detector dye at the 5' end and with a quencher at the 3' end hybridises to a specific sequence between the two primers. The annealing temperature of the probe is higher than the melting temperature of the primers, guaranteeing that in every cycle a labelled probe hybridizes to every complementary sequence before primers bind. After the primers have bound to the DNA, the Taq polymerase starts elongation and at the same time, owing to its nuclease activity, nicks the 5' end of the probe. This separates the detector from the quencher, which causes the detector dye to emit a characteristic fluorescence signal when excited by light. Because the cleavage of each dual labelled probe is the result of a single amplification event, the intensity of excited fluorescence is directly

proportional to the number of probes cleaved and the amount of PCR product synthesised..

It has recently been proven, that the accuracy of the Real Time qPCR is even superior to the specifications given by equipment manufacturers, in that it can detect as low as 3:2 differences in copy number, whereas the lowest differences reported to date were 2:1. Detection of the differences of the order of 3:2 is evidently a requirement when attempting to determine fetal aneuploidy. Achieving such high accuracy requires implementation of stringent experimental and analytical conditions. The quantitative PCR is based on comparable and stable PCR efficiencies for the target sequences, one located e.g. in the Down's syndrome critical region and the others on different chromosomes.

Currently spectral overlap of fluorophores limits the maximum possible number of simultaneous amplifications up to four sequences in existing Real Time qPCR machines, so potentially four sequences could be quantitated in a single test in one well.²⁶

There are significant advantages of Real Time qPCR in comparison to other systems: the closed system reduces the risk of contamination and saves space. There is no need for post PCR processing, which saves time and provides an elegant and reproducible assay. Data for analysis are collected not at the arbitrarily chosen end of the experiment, but accumulate during the amplification, and the optimal point (or multiple points) for analysis may be chosen retrospectively, depending on the characteristics of the amplification profile. The shape of the amplification curve per se provides means of quality control of the analysis. The technique is amenable to automation and high throughput and the sample demand is little. Also, as it targets non-polymorphic sequences, a once established assay should be applicable to all patients, and multiple loci only need to be examined for security purposes.

However there are some disadvantages too: the cost of the reagents surpasses the conventional assays and only up to four sequences can be examined in multiplex. The fact, that the control sequences come from other chromosomes of the same sample implies that real-time quantitative PCR cannot distinguish triploidy 69,XXX from normal 46,XX.

Discussion

We are still at an early stage of development of molecular techniques for the detection of fetal aneuploidy. We may even hypothesize, that trustworthiness of classical cytogenetics hampered the development of competitive molecular methods. Recently the main source of increased demand for faster, molecular methods is a large proportion of fetuses that are karyotyped because of the very high risk after efficient non-invasive screening. Up to now however, most prenatal laboratories do not perform these tests in all patients and there is no uniform agreement which of these methods will become a method of choice.

DNA sequence based methods provide a means for the detection of autosomal trisomy in one of the following ways:

- Detection of polymorphisms. Making use of highly polymorphic sequences, detection of three allelic variants of a sequence in a sample indicates trisomy of the putative chromosome. Detection of two allelic polymorphic sequences does not exclude trisomy. If one of them is present in a second copy from a surplus chromosome, detection of a 2:1 ratio is possible, denoting trisomy. Methods based on this phenomenon PCR of STR, melting analysis of SNP
- Detection of abnormal sequence or chromosome dosage is based on the number of sequence copies specific for the target chromosome relative to another sequence on another chromosome. This approach requires precise quantitation, because the proportion of target sequence copy number in the trisomic cells is 3:2 – euploid is 2:1. Methods using this are real-time PCR, MLPA, MAPH,

It seems probable that the choice of the molecular method for the detection of aneuploidy will depend on the indications for the test. If such test is to be performed in a low risk patient just for early reassurance, screening of the sequences only from the chromosomes that are most often engaged in aneuploidy would be recommended. In a patient at high risk of aneuploidy because of suspicious US/biochemical findings a more detailed analysis, comprising also common microdeletion syndromes, would seem justified. In future, molecular methods could also screen the subtelomeric regions, if current presumptions about their association with mental retardation are confirmed.

Current molecular methods do not provide a means for genome-wide-screening, although their resolution is considerably superior to classical cytogenetics. At present maximally about 40 sequences can be examined in one test, which does not compare favorably with the figure of 500 bands, easily achievable by classical cytogenetics, which simultaneously surveys ploidy of all chromosomes, whereas analysis directed at specific alleles is constrained to the targeted regions. The molecular methods however concentrate on particular “hot spots” i.e. critical regions, associated with specific, relatively common pathologies, their results are unequivocal, and lesions beyond the resolution of cytogenetics may be detected. The future therefore seems to belong to the molecular approach.

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Novel Real-Time Quantitative PCR Test for Trisomy 21.

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Chapter 4

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The detection of gross chromosomal abnormalities is a major focus of invasive prenatal diagnosis testing, of which the most common cytogenetic anomaly in live births is trisomy 21 (Down syndrome). Classically these examinations are lengthy procedures relying on karyotypic analysis of cultured amniocytes or chorionic mesenchyme. More rapid alternatives, such as fluorescence in situ hybridization and quantitative fluorescent PCR on uncultured cells, are time- and labor-intensive. Here we describe a novel real-time PCR (1, 2) assay for the detection of trisomy 21 that is readily amenable to automation and high-throughput screening.

Real-time PCR can determine subtle alterations in gene dosage, such as hetero- or homozygosity of the RhD locus (3) and protooncogene amplification in breast cancer patients (4). We examined whether a real-time PCR assay could determine chromosomal ploidy, in particular for the prenatal detection of Down syndrome (trisomy 21). We examined the coamplification of a genetic locus (amyloid gene) in the Down's region of chromosome 21 and a control locus [glyceraldehyde 3-phosphate dehydrogenase (GAPDH)] on chromosome 12. The amyloid gene locus on chromosome 21 was chosen because aberrant expression of this protein has been implicated in the physiologic lesions associated with Down syndrome (5). Furthermore, use of this locus should enable the detection of unbalanced Robertsonian translocations involving the Down's region of chromosome 21, which occur in ~ 4% of Down syndrome cases (6). No such cases were available during in this pilot study.

We used a multiplex real-time PCR assay in which amplification of both loci was simultaneously monitored in the same reaction vessel. In this way, we could be sure that any alterations were not attributable to well-to-well variation. The following primers and probe were used for the amyloid gene on chromosome 21: forward, 5'-GGG AGC TGG TAC AGA AAT GAC TTC-3'; reverse, 5'-TTG CTC ATT GCG CTG ACA A-3'; and probe, 5'-(FAM) AGC CAT CCT TCC CGG GCC TAG G (TAMRA)-3', where FAM is 6-carboxyfluorescein, and TAMRA is 6-carboxytetramethylrhodamine. The sequences for the GAPDH primers and the probe were as follows: forward, 5'-CCC CAC ACA CAT GCA CTT ACC-3'; reverse, 5'-CCT AGT CCC AGG GCT TTG ATT-3'; and probe, 5'-(VIC) AAA GAG CTA GGA AGG ACA GGC AAC TTG GC (TAMRA)-3'. In the TaqMan analysis, we used 25- μ L reaction volumes containing 2 μ L of the extracted DNA, 300 nmol/L each of the primers, 150 nmol/L each of the dual-labeled TaqMan probes, and further components supplied in the TaqMan Universal PCR Master Mix (Perkin-Elmer),

corresponding to 3.5 mmol/L MgCl₂, 100 µmol/L dNTPs, 0.025 U/µL AmpliTaq Gold, and 0.01 U/µL Amp Erase. Cycling conditions were as follows: incubation for 2 min at 50 °C, to permit Amp Erase activity, and for 10 min at 95 °C for AmpliTaq Gold activation and DNA denaturation, followed by 40 cycles of 1 min at 60 °C and 15 s at 95 °C.

In proof-of-principle experiments we examined DNA extracted from amniocyte cultures obtained from 10 trisomy 21 fetuses. As controls we used DNA samples obtained from 11 apparently healthy individuals. These samples included peripheral blood as well as amniocyte cultures. DNA was extracted from 400-µL samples, using the QIAamp DNA Blood Mini Kit according to the manufacturer's recommendations (Qiagen). We included several dilutions of these DNA samples because we were concerned that the assay should be applicable over a broad range of DNA concentrations. In this small series of experiments we were able to show that the ratio of the two loci in these samples, as determined by the difference in threshold cycle value (C_T), distinguished trisomy 21 from karyotypically normal tissue (Fig. 1). Furthermore, we ascertained that clear segregation of karyotypically normal and trisomic samples was possible over a wide sample concentration range, in that we still observed optimal results when we used 10-fold diluted DNA samples and DNA concentrations as low as 10 mg/L.

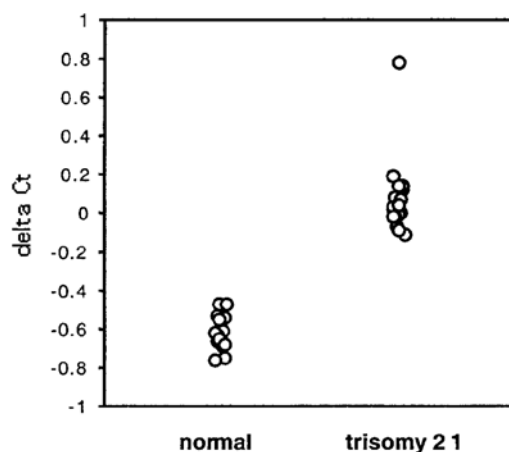


Figure 1. Clear segregation of trisomy 21 samples from those with a normal karyotype by a multiplex real-time PCR assay that is able to accurately assess the ratio for a chromosome 21-specific locus and a control locus on chromosome 12.

Differences in threshold cycle numbers (ΔC_T) greater than +0.2 are indicative of a trisomy 21 karyotype, whereas differences less than -0.4 indicate a normal karyotype.

We required that the following two conditions be met: that efficient amplification of both loci examined occurred and that both loci were amplified with equal efficiency over the entire exponential phase of the PCR. For this purpose we examined, on the one hand, the amplitude of the amplification curve (R_N). Because this parameter is indicative of amplification efficiency and template concentration, it can be considered a rather basic endpoint measurement for quality surveillance. In our pilot study we arbitrarily selected a R_N cutoff value of 0.7. It is possible that another cutoff value may be obtained in a larger series of experiments. For the second step, which was to ensure that both loci had been amplified with equal efficiency, we examined their ratio at three separate points (bottom, middle, top) along the exponential phase of the amplification reaction. Samples in which a deviation was found were disregarded.

Using this approach, we were able to correctly determine the ploidy in 9 of 11 cases with normal karyotype. Our analysis included six diluted samples in which the ploidy was also identified correctly. In the 10 samples from trisomy 21 fetuses, we were able to determine the ploidy correctly in 9 samples and in 10 separate dilutions. In those cases where we could not reliably determine fetal ploidy, we were alerted to incorrect amplification either by a R_N value ≤ 0.7 or by an inconsistency in the ratio of the two loci examined at the three thresholds selected over the exponential phase of the amplification. In one case of trisomy 21, an outlier was recorded with a C_T of almost 0.8. This sample was almost 3 years old. Interestingly, in two dilutions of this extraction, we obtained the expected C_T values for trisomy 21.

These added precautions prevented incorrect determination of the ploidy in this series. A recent study regarding the detection of single-nucleotide polymorphisms by real-time PCR indicated that relying solely on C_T values may lead to erroneous results, which could be averted by examining the corresponding R_N values (7). Our smallscale pilot study, therefore, suggests that real-time PCR technology can be used for the rapid determination of trisomy 21. This technology could be easily extended to examine the most common fetal aneuploidies (13, 16, 18, X, and Y). Because real-time PCR permits the analysis of numerous samples in an automated manner, this technology may be more suited to this task than current molecular or cellular cytogenetic methods because these are considerably more time- and labor-intensive. In a largescale setting, this method may also compare favorably with a fluorescent PCR-based approach or fluorescence in situ hybridization analysis regarding speed and price.

We caution against premature use of the described method for routine prenatal diagnosis of trisomy 21. The feasibility of this method must be explored in a large-scale prospective study before it is applied to a routine diagnostic setting.

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Determination of RHD Zygosity Using Real-Time Quantitative PCR.

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Chapter 5

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Summary

At present RHD incompatibility is still an obstetric problem despite prophylactic treatment. A very welcome recent technical advance has now made it possible to determine the foetal RHD status in a non-invasive risk-free manner using cell free foetal DNA in maternal plasma. In some cases, however, where there is a high risk that the foetus may be affected by HDN (haemolytic disease of the newborn), it may be of interest to determine whether the father is hetero- or homozygous for the RHD gene, since in the former instance there is only a 50% chance that the pregnancy is affected. It has recently been shown that quantitative PCR assays, in particular real-time Taqman PCR, can be used to determine the RHD gene dosage, and also to determine foetal aneuploidies. We demonstrate that the same real-time Taqman PCR assay we had previously developed for non-invasive analysis of the foetal RHD gene and the foetal Y chromosome from maternal plasma can be used to determine the paternal RHD genotype.

Key words: *RHD; paternal genotype; prenatal diagnosis; real-time PCR*

The determination of RHD zygosity has until recently been a tedious procedure and is usually inferred from the serotype, family history and / or complex PCR-based RFLP assays [1–3]. To address this issue, two recent publications have shown that quantitative PCR assays can be used to accurately genotype the predominant Caucasian RHD locus. In the first instance, Chiu and colleagues determined RHD zygosity using a real-time Taqman PCR assay in which the dosage of the RHD gene was compared with a control locus, namely the albumin gene [4]. In the second approach, described by Pertl and colleagues, a quantitative fluorescent PCR assay compared the dosage of the RHD gene to that of the related RhCE gene locus [5]. A further development of the real-time PCR approach by our group has shown that this technology is sufficiently sensitive to detect even smaller differences in gene dosage, namely those which occur in foetal aneuploidies, in which instance only a 50% increase in gene copy number occurs and not 100% as is the case for the RHD gene [6].

Since it is only of interest to determine the RHD genotype of the male partner (the mother by definition being RHd), we were curious as to whether a real-time Taqman PCR assay we had previously developed for another purpose, non-invasive risk-free determination of foetal RHD status and sex from maternal plasma [7], could be used for the determination of RHD zygosity.

The Taqman® real-time PCR assay centres upon the detection of a fluorescent signal generated from the cleavage of a target sequence specific probe by the Taq polymerase during each cycle of the PCR reaction [8]. As this signal is directly proportional to the PCR product being amplified, it permits very precise quantitation of the amount of initial input template. This is ascertained from the so-called threshold cycle, also termed the C_T value, the point where the exponential phase of the amplification curve crosses a defined threshold line. As this C_T value is a reflection of the number of PCR cycles required to reach this threshold, the lower the C_T value is, the higher is the concentration of input target template.

By using a real-time PCR assay in which two genetic loci are amplified simultaneously in a multiplex reaction, it is possible to determine the relative ratio of these two loci by subtracting their respective C_T -values, e.g.:

$$\Delta\Delta C_T = C_T(\text{target A}) - C_T(\text{target B}) = C_T(\text{RHD}) - C_T(\text{SRY})$$

Since 1 cycle entails a doubling of the PCR product, the ratio of RHD: SRY = $2^{-(\Delta\Delta C_T)}$. Hence, if both the RHD and SRY genes are present with the same gene dosage, e.g. 1 copy (heterozygous RHD/RHd), the difference in threshold cycle number ($\Delta\Delta C_T$) will be 0 cycles, whereas if 2 copies of the RHD gene are present (homozygous RHD/RHD) the difference in threshold cycle number ($\Delta\Delta C_T$) will be 1 cycle. It should be noted that these are theoretical values and that slight differences are bound to occur due to minor variations in the PCR assay, especially if one reaction proceeds with slightly greater efficiency than the other.

These minor deviations are bound to occur even if considerable care has been taken to optimise the paired PCR reactions in such a manner that no significant difference is discernible between their efficiencies, as measured by the slope of the PCR assays [4, 6].

Precautions which need to be taken in order to obtain a correct result include the use of multiplex PCR reactions whereby both target gene sequences are analysed simultaneously in the same reaction vessel, as well as the inclusion of genotypically defined samples in each analysis [4, 6, 9]. By using such precautions it has previously been shown by Chiu and colleagues [4] that realtime PCR can be used to determine the paternal RHD genotype. More recently we have shown that real-time PCR can also be used to detect more subtle increments in gene dosage (only 50%), such as those occurring in foetal trisomies [6].

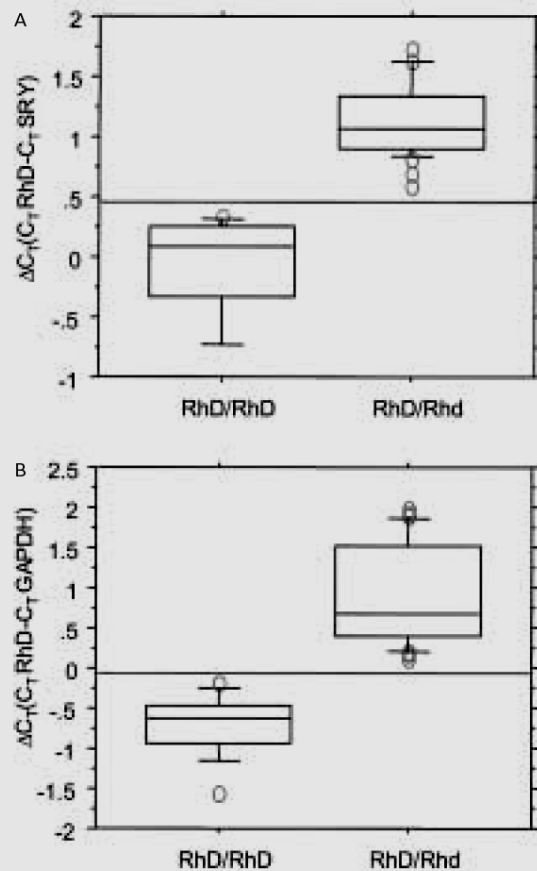
To test the possibility that the real-time PCR assay we had previously developed for analysis of the foetal RHD and SRY genes in maternal plasma [7] could be used to determine the paternal RHD genotype, we examined 39 DNA samples obtained from males who had been serologically typed to be RHD (Swiss Red Cross Blood Bank, Basel, Switzerland). To confirm the accuracy of our assay we also determined RHD zygosity in these samples using a modification of the real-time PCR described by Chiu and colleagues [4]. In our investigation the dosage of the RHD gene was compared to another control locus, which in our case was the GAPDH (glyceraldehyde 3-phosphate dehydrogenase) gene and not the albumin gene. The reason for this modification was that we have previously described the use of the Taqman real-time PCR assay for this GAPDH control locus in a number of studies, using either cell free DNA [7, 10] or genomic DNA [6]. In our analysis, all samples were run in triplicate.

The results of our analysis indicated that the samples in our investigation clustered into two specific groups, one having a median of almost 0 and the other of approximately 1 (see Figure 1A).

To ascertain the genotype of these two groups we also tested our samples using a modification of the assay described by Chiu and colleagues [4], whereby we compared the dosage of the RhD gene with that of the GAPDH gene [6]. Here we would expect an opposite pattern, as the GAPDH gene is normally present in 2 copies, i.e. the RHD/RHD genotype should yield a $\Delta\Delta C_T$ value of 0, whereas the RHD/RHd genotype should differ by 1 cycle from the C_T value for the GAPDH reference. In this analysis we again found that the samples clearly clustered into two groups, one with a median $\Delta\Delta C_T$ value of approximately -0.5 and the other a median $\Delta\Delta C_T$ value of approximately 0.5 (see figure 1B). It is of interest that a 100% concordance was observed between the 2 groups of samples in both assays.

Figure 1

Box-plot showing the discernment of RHD zygosity by real-time PCR. In Figure 1A, the RHD gene has been examined in comparison with the SRY gene. In Figure 1B, the RHD gene has been examined in comparison with the GAPDH gene. The lines inside the boxes indicate the median value, whereas the upper and lower limits of the boxes represent the 75th and 25th percentiles respectively. The upper and lower horizontal bars indicate the 90th and 10th percentiles respectively. Outliers are indicated by open circles. The X axis indicates the RHD genotype determined, whereas the Y axis indicates the pertinent ΔC_T values. No overlap was observed between the two genotypic groups. The cut-off values are indicated by stippled lines.



Interpretation of the results, however, is not as straightforward as it would seem, due to a significant deviation from the expected $\Delta\Delta C_T$ values. In this manner, even though the expected $\Delta\Delta C_T$ values in the first SRY:RHD assay would be -1 for the RHD/RHD genotype and 0 for the RhD/Rhd genotype, it is apparent that the PCR reaction for the SRY gene has proceeded with slightly better efficiency than that for the RHD gene. Consequently the expected RHD:SRY $\Delta\Delta C_T$ value in the case of the RHD/RHD genotype has shifted up from the theoretical value of -1 to almost 0 , whilst that for the RHD/Rhd genotype has similarly also been shifted up by 1 cycle from the theoretical value of 0 to almost 1 .

In an analogous manner the GAPDH PCR reaction has proceeded slightly more efficiently than that for the RHD gene, resulting in a shift in the expected RHD:GAPDH $\Delta\Delta C_T$ value. In this case the $\Delta\Delta C_T$ value for the homozygous RHD/RHD genotype has shifted down by almost half a cycle from the theoretical value of 0 to almost -0.5 . Equally, that for the heterozygous RHD/Rhd genotype has been shifted down by 0.5 cycles from the theoretical value of 1 to almost 0.5 .

We have previously indicated that to counter these unwanted shifts it is possible to use $\Delta\Delta C_T$ values, whereby the C_T value of the sample being analysed is compared to a mean C_T value comprised of the analysis of a large number of samples of known genotype [6, 9]. Furthermore, these drifts in $\Delta\Delta C_T$ values stress the importance of including samples of known genotype in each analysis, to counter inter-run variations [4, 6, 9]. It is also imperative to analyse the samples in a multiplex manner whereby both target genes are analysed simultaneously in the same reaction vessel, and not to attempt this type of analysis by comparing the assessed gene dosage against a standard curve [4, 6, 9].

An important feature that is evident from our analysis is that the two groups can be segregated with 100% accuracy by the use of particular cutoff values. With regard to the RHD/SRY assay, we determined that a cut-off $\Delta\Delta C_T$ (C_T RHD $- C_T$ SRY) value of 0.5 could be used to distinguish between the heterozygous (RHD/Rhd) and homozygous (RHD/RHD) genotypes, in that the values for the RHD/Rhd genotype had $\Delta\Delta C_T$ values which clustered around 1 , while RHD/RHD genotype had $\Delta\Delta C_T$ values which clustered around 0 (Figure 1A). No overlap between the two groups was found to occur.

Similarly, for the RHD/GAPDH assay, a $\Delta\Delta C_T$ (C_T RHD $- C_T$ GAPDH) cut-off value of 0.0 could be used to distinguish the homozygous RHD/RHD genotype from the

heterozygous RHD/ RHd one. In this test the heterozygous (RHD/ RHd) genotypes have $\Delta\Delta C_T$ values above 0.0, while the homozygous (RHD/RHD) genotype had $\Delta\Delta C_T$ values below 0.0 (Figure 1B). Once again, no overlap between the two groups was found.

As explained previously, the reason for the difference in the $\Delta\Delta C_T$ cut-off values between these two assays is that for the RHD/GAPDH assay the RHD gene is compared with both alleles of the GAPDH gene on chromosome 12, whereas for the RHD/SRY assay, the dosage of the RHD gene is compared to the single SRY allele on the Y chromosome.

The validity of our analysis is underscored by our subsequent examination of 3 samples known to be from RHD heterozygous males, in which case we were able to determine the correct genotype in a blinded manner (data not shown).

In the sample cohort of 39 samples we determined that 26 (66%) were heterozygous for the RHD gene (RHD/RHD) and 13 (34%) were homozygous (RHD/RHD). Once again there was complete concordance between the 2 assays. Although the expected frequency of RHD heterozygosity would be 56% [1], it is probable that our results are slightly skewed by the small number of cases examined.

Although our data obtained by the use of two independent real-time PCR assays do serve to indicate that this technology can potentially be used for the determination of RHD zygosity, we have also shown that the employment assays and their subsequent analysis require considerable experience if a correct diagnostic outcome is to be achieved. Furthermore, the data also emphasise the importance of running genotypically well defined control samples in parallel with the sample being analysed, as the theoretically expected $\Delta\Delta C_T$ values cannot be used. Our study also indicates the usefulness of running two independent analyses in parallel as a potential safeguard against erroneous results, a feature we have also observed previously when attempting to discern foetal trisomies by the use of real-time PCR [6, 9].

Despite these promising results we caution against the premature clinical use of these assays, since their efficacy has not yet been determined in large scale studies, nor is it yet known how they may be influenced by the RHD polymorphisms [1–3] prevalent in many ethnic populations.

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Use of Real-Time PCR for the Detection of Fetal Aneuploidies.

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Chapter 6

Contribution to: Clinical Applications of PCR, 2nd Edition
At the Editor: YM Dennis Lo

Abstract

With the advent of real-time PCR it is now possible to measure nucleic acid concentrations with an accuracy that was not deemed possible only a few years ago. Examples are the analysis of gene expression or gene duplications / losses, where two-fold differences in nucleic acid concentration have routinely been determined with almost 100% accuracy. As our primary interest is in prenatal diagnosis, we have investigated whether real-time PCR could be used for the diagnosis of chromosomal anomalies, in particular the aneuploidies such as trisomy 21, where the difference in copy number is only 50 %. The feasibility of such an approach was first tested in a pilot study, in which we were able to demonstrate that trisomy 21 samples could be detected with 100 % specificity. We have recently modified this test in order to permit the simultaneous analysis of trisomies 18 and 21 and have in a large scale analysis demonstrated that our approach can be used for the highly reproducible and robust detection of only 1.5 fold differences in gene copy number.

Keywords

Multiplex real-time PCR, relative quantification

Prenatal diagnosis

Amniotic fluid, amniocentesis

trisomy 21, Down's Syndrome, trisomy 18, karyotype, aneuploidy

Genetics, DNA analysis, gene copy number

Molecular probes, PCR primers, PCR kinetics

1. Introduction

1.1 Background

The detection of gross chromosomal abnormalities is a major focus of prenatal diagnostics, of which the most common cytogenetic anomaly in live births is trisomy 21, also known as Down's Syndrome. Other fetal aneuploidies frequently detected involve chromosomes 13, 16, 18 and both sex chromosomes (X and Y). Currently, prenatal diagnosis of genetic anomalies relies on invasive procedures such as amniocentesis and chorionic villus sampling (CVS), from which the full fetal karyotype is usually determined using cultured cells. The two weeks period needed for cultivation and subsequent analysis has proven to be associated with considerable parental anxiety and medical problems in those situations requiring therapeutic intervention.

In order to address these needs, more rapid methods for the prenatal diagnosis of fetal chromosomal aneuploidies have recently been developed and implemented. The first of these to be commercially introduced was multi-colour fluorescence in-situ hybridisation (FISH) for uncultured cells. Although this method is very reliable and has proven in large-scale studies to be very accurate, it is a time and labour intensive procedure. Furthermore, as this method requires intact cells, it can only be used on fresh or specially stored samples [1-4]. The next method that has seen widespread clinical application, particularly in the UK, is quantitative fluorescent PCR analysis of short tandem repeats (STR) [5-8]. This method has also proved itself to be rapid and reliable, once the initial problems with polymerase stuttering and amplification failure of the highly repetitive loci had been overcome.

An important point to bear in mind regarding both of these rapid diagnostic tests is that they currently only permit a result regarding the most common fetal aneuploidies (chromosomes X, Y, 13, 18 and 21). Hence, it is still necessary to resort to the normal 2-week cell culture-based analysis in order to obtain a full karyotype. Maternal blood contamination of the amniotic fluid or chorionic villus sample, although infrequent (less than 2% of samples collected), can interfere with either method of analysis, as the results cannot be distinguished from cases of fetal mosaicism.

The recent development of real-time PCR has rapidly emerged as a powerful tool for the accurate and precise determination of template copy numbers, and has found wide-spread applicability in the analysis of gene expression, cell free DNA in body fluids, as well as for measuring gene duplications or deletions in cancer research [9-13]. The precision of current assays and technology, however, was not thought to permit analyses of less than two fold differences of target template concentrations.

As our primary interest is in the development of new methods for prenatal diagnosis, we were interested in whether this new technology could be used for the determination of fetal trisomies. This would, however, entail the resolution of less than 2 fold increments in target gene copy number, as in these instances there is only a 50% increase present in the amount of a particular chromosome.

The reason for choosing a real-time “Taqman” PCR approach is that we have considerable experience with the methodology [14-17], especially in the quantitation of trace quantities of cell free fetal DNA in the maternal circulation. To test the feasibility of this approach we first performed a small-scale study involving trisomy 21, as this is the most common aneuploidy in live births. In our study we compared the gene dosage of a sequence on the Down’s critical region on chromosome 21 to a control locus on chromosome 12. Our analysis indicated that by using such an approach of comparative quantitation it was indeed possible to discern karyotypically normal samples from trisomic ones, provided that certain criteria concerning quantity and quality of DNA and replicate reaction uniformity were met [18]. After successful completion of this pilot study, we have now extended this method for the simultaneous detection of trisomy 18 and trisomy 21. In this analysis the two chromosomes being interrogated are quantified relative to each other.

Future developments would include the development of a test in which the other common fetal aneuploidies (chromosomes 13, 16, X, Y) would be analysed in a matrix-type assay. By using such a matrix approach, whereby the dosage of each chromosome is measured relative to the other chromosomes, an automatic system of “checks and balances” would be implemented, thereby helping to reduce the potential error rate considerably.

The use of the real-time PCR assay need not be restricted solely to the detection of aneuploidies, but can also be applied for the discernment of translocations and for the distinction of these from chromosomal trisomies. This can be achieved by amplifying sequences critical to the region translocated, such as those located in the Down’s

region of chromosome 21, which should enable the detection of those unbalanced Robertsonian translocations which occur in approximately 4% of Down's syndrome cases. It has to be noted however, that the design of the assay does not permit to detect triploidies, since the chromosomal balance is equal to a normal karyotype.

Advantages of the real-time PCR test for prenatal diagnosis are that it is insensitive to maternal blood contamination of samples where the maternal DNA is present at a small percentage (about 20% or less of the total DNA in the sample). On the other hand, this test will not be able to detect low level chimerism. Akin to the PCR amplification of STRs, the real-time PCR test also can be used on a wide variety of sample materials and does not require fresh or specially stored cells, since it only requires genomic DNA.

Furthermore, the assay is readily amenable to automation, and by making use of the current real-time PCR 96 or 384 well formats, it also facilitates high throughput. In addition, by making use of a closed system, whereby the samples are analysed directly in the PCR reaction vessel during the amplification and do not need to be opened for analysis as is the case for the analysis of STRs, the assay is also less prone to contamination.

1.2 The principle of relative quantification by real-time PCR in relation to the determination of chromosome ploidy.

Real-time PCR is based upon the detection and quantitation of a fluorescent signal that is directly proportional to the PCR product being generated during each cycle of the PCR. In our assay the fluorescence is generated by the 5' nuclease method: A so called TaqMan probe hybridises specifically to one strand of the DNA sequence between the two primers. The probe is labelled with a fluorescent detector dye at its 5' end and with a quencher dye at its 3' end. The annealing temperature of the probe is 10 °C higher than the annealing temperature (T_A) of the PCR reaction, which is the melting temperature of the primers. This ensures that in each cycle of the reaction a probe binds to every target sequence before primers anneal. When the primer binds to the target, the Taq polymerase immediately starts extending the primer and by its nuclease activity cleaves the 5' end of the probe. This nucleolytic activity separates the detector dye from the quencher dye, thereby permitting the detector dye to emit a

characteristic fluorescence signal when excited by an appropriate light source. As the displacement of each probe molecule is the result of a single template amplification, the amount of fluorescent signal measured is therefore directly proportional to the number of probes cleaved and the amount of PCR product synthesised.

For the correct analysis of such real-time PCR assays several parameters have been devised. The first of these is the C_T -value, or threshold cycle, is defined as the cycle number at which point the amplification curve crosses the threshold line in semi-log view of the amplification plot (refer to figure 1).

Although the C_T -value is the chief factor used in most real-time PCR analyses, in our experience another important parameter, especially for the analysis of discrete template increments, is the normalised final fluorescence, ΔR_n . This ΔR_n -value, a measure of the accumulation of specific fluorescence, is indicative of the amplification efficiency and initial template concentration (refer to figure 1).

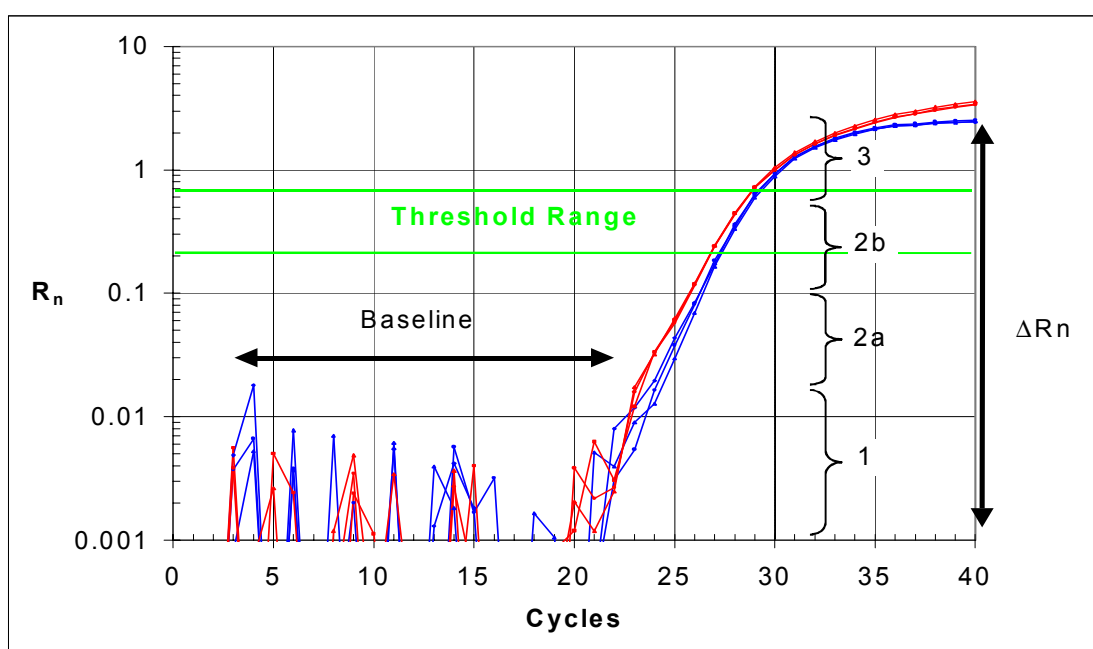


Figure1. Amplification plot of the real-time PCR data. 1: Phase of observed background fluorescence. 2: Observable exponential phase. 2a: Measurements are close to the detection limit. Due to the high contribution of the background fluorescence to the total fluorescence measured, replicate curves can deviate (as seen in the blue curves). 2b: Influence of background fluorescence minimal. 3: Linear and plateau phases of the amplification with decreasing amplification efficiencies.

Displayed are triplicate amplifications of a sample with trisomy 21. The red curves represent data from chromosome 21 (FAM dye), the blue curves from chromosome 18 (VIC dye).

As our real-time PCR test for fetal aneuploidy entails the simultaneous amplification of two chromosomal loci (e.g. chromosome 21 vs. chromosome 12 or chromosome 21 vs. chromosome 18) in a multiplex reaction, their product formation is detected by two different fluorescent dyes. As each of these reporter dyes has a discretely different emission spectrum, and as the amount of each dye measured is proportional to the relevant target template, this enables the relative quantification of both chromosomes.

This can be calculated by using the ΔC_T , which is the difference of the C_T -values of the first (e.g. chromosome 18, VIC dye) and the second amplified sequence (e.g. chromosome 21, FAM dye) in one reaction well (refer to figure 2):

$$\begin{aligned}\Delta C_T &= C_T (\text{target A}) - C_T (\text{target B}) \\ &= C_T (\text{VIC, Chromosome 18}) - C_T (\text{FAM, Chromosome 21})\end{aligned}$$

In order for this analysis to be accurate it is important that the co-amplification of two sequences occurs in the same reaction vessel, this guarantees that no well to well variation occurs between the two amplified targets [19]. In this regard, it is important to realise that the analysis cannot be carried out in a singleplex manner. As any small well to well deviations resulting from minute pipetting errors, alterations in polymerase activity, temperature or illumination gradients, as well as unequal reagent depletion, will lead to an inaccurate assessment of the target chromosome ploidy. In a similar manner it is not possible to determine the ploidy of a sample by referring to a standard curve, as this analysis will be misled by the same factors affecting singleplex analyses, a facet which is not alleviated by post-run data analysis [22-25].

In order to balance small fluctuations and ensure that the amplification of both target sequences has proceeded with similar efficiency, we have devised the following analytical aid: instead of a solitary C_T -value, 3 to 4 points are chosen along the linear phase of the amplification plot (refer to figure 2). Those samples in which a deviation is found to occur at these points are either discarded or re-analysed. If this proviso has been made, then the fluorescent signals from both amplifications will be detected simultaneously if the sample is karyotypically normal i.e., both chromosomes being interrogated have the same copy number (refer to figure 3). In case of a trisomy, the

chromosome present at 3 copies per cell will be detected at a lower (earlier) C_T -value than the other will.

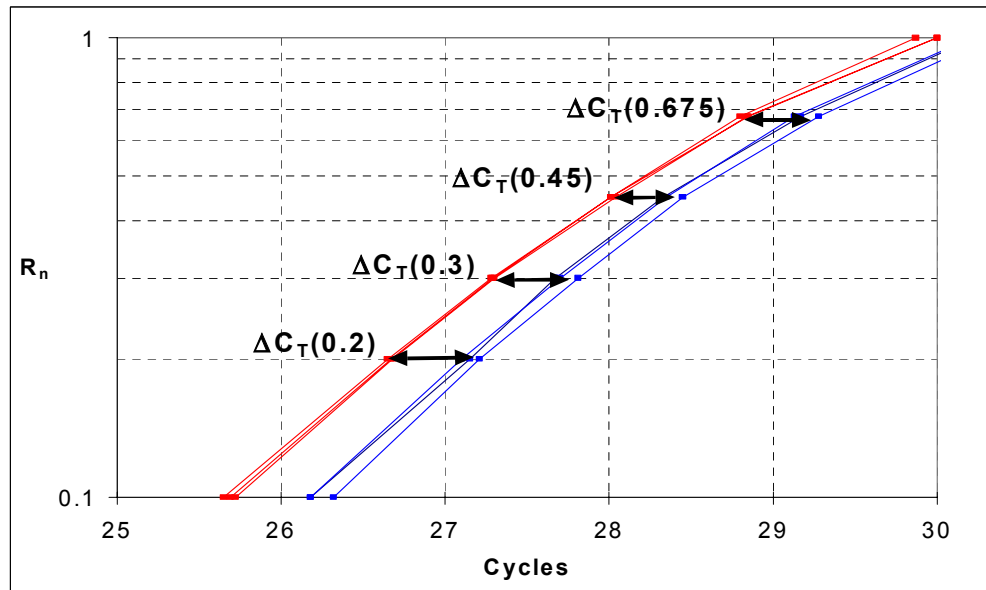


Figure 2. ΔC_T in the amplification plot (magnification of figure 1). The ΔC_T s of the four thresholds, indicated by double-headed arrows.

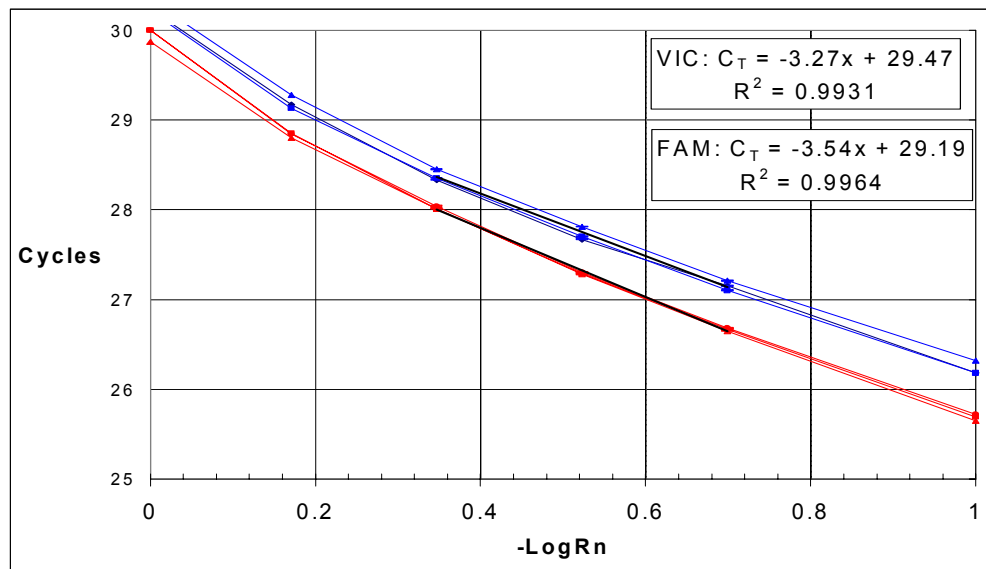


Figure 3. Linear regression of fluorescence data: The C_T -values of the trisomy 21 sample measured in triplicate for thresholds 0.2, 0.3 and 0.45 were fitted in a linear regression [23]. The slope for the amplification of chromosome 18 (VIC dye) is -3.27 , the slope for chromosome 21 (FAM dye) is -3.54 . The amplification efficiencies ($E = 10^{(1/\text{slope})-1}$) for both chromosomes are greater than 0.90 but clearly different with this method of efficiency determination: E (chromosome18, VIC) = 1.02 and E (chromosome 21, FAM) = 0.92. This can be a result of the probe and dye properties or the “ C_0 t effect” [26]. With the standard curve method, the amplification efficiencies determined are equal.

The measured differences in C_T (the ΔC_T) between the two target chromosomes can be converted into a ratio [20]:

$$\text{target A / target B} = 2^{-(\Delta C_T)} = \text{Chromosome 18 / Chromosome 21}$$

In theory the difference in threshold cycle number (ΔC_T) for a normal (and for a triploid) karyotype will be 0 cycles, for a trisomic sample it will be ± 0.58 cycles, since $2^{-(0.58)} = 1.5$

To correct for slight differences in reaction efficiencies and detector dye intensities, the $\Delta\Delta C_T$ method can be used which relies on the analysis of a reference or calibrator sample [21]. Normally the reference or calibrator sample is a sample of known (preferably normal) karyotype. The ΔC_T of the reference sample is subtracted from the ΔC_T of the sample, and then the corrected ratio of the target sequences in the sample can be calculated as follows:

$$\begin{aligned}\Delta\Delta C_{T \text{ calibrated}} &= \Delta C_T (\text{sample}) - \Delta C_T (\text{calibrator}) \\ (\text{target A / target B})_{\text{calibrated}} &= 2^{-(\Delta\Delta C_{T \text{ calibrated}})}\end{aligned}$$

Even though this method is more reliable than solely relying on the ΔC_T -value(s) of the analysed sample, the use of a single reference sample can nevertheless introduce a small source of error. This may be caused by minute deviations in the amplification and signal generation, which may in turn lead to the misinterpretation of the sample analysed. In this regard it is worth noting that a seemingly small shift in the reference ΔC_T -value of only +0.15 cycles from its “real” value may result in misdiagnosis of a sample with a similarly small deviation of -0.15 cycles.

For this reason we recommend that for the reliable determination of ploidy an adaptation of the $\Delta\Delta C_T$ method be used, whereby the samples being analysed are quantified relative to a reference ΔC_T -value determined from the average ΔC_T -values of all karyotypically normal samples tested previously. Furthermore, if this analysis suggests that a particular sample is aneuploid, then an added precaution may be to analyse this sample using a reference comprised of similar aneuploid samples. In this manner an opposite pattern to that normally expected will emerge, in that the $\Delta\Delta C_T$

analysis using a normal ploidy reference should be close to the theoretical value of ± 0.58 cycles, whereas the $\Delta\Delta C_T$ comparison using a matched aneuploid reference standard should be close to 0.

In reality, however, the experimentally measured differences in $\Delta\Delta C_T$ between normal and trisomic karyotypes is not 0.58, but ranges from 0.47 to 0.55 depending on threshold-value set and the type of trisomy. As a consequence the calculated chromosome ratios will average 1.4 rather than 1.5 for a trisomic sample. Although the exact reason for this small anomaly is unclear, it is likely to be attributable to minor differences in amplification efficiencies or incomplete spectral separation of the dyes [26].

Since the $\Delta\Delta C_T$ -values themselves are indicative of the karyotype i.e. close to 0 for normal and ± 0.58 depending on the type of trisomy, it is not actually necessary to determine the ratio. This is particularly evident when taking an average $\Delta\Delta C_T$ -value taken at the 4 independent threshold points (refer to table 2). Alternatively it is possible to add these 4 independent $\Delta\Delta C_T$ -values. In this manner, a normal karyotype will have a value close to 0, whereas a trisomy 21 sample will be of the order of 2 and a trisomy 18 sample will have a value of approximately -2.

In this manner it is possible to determine the samples' karyotype with a minimum of post-run work by setting the cut-off range around the average $\Delta\Delta C_T$ of a given karyotype. In our analyses, we therefore, set a cut-off range of 0.25 cycles around the average measured $\Delta\Delta C_T$ of a given karyotype, since the measured $\Delta\Delta C_T$ s between normal and trisomic are around ± 0.5 . In this regard, the $\Delta\Delta C_T$ calibrated of karyotypically normal samples will have a range of 0.00 ± 0.25 . The accuracy of the test result can be confirmed by re-analysing the suspected trisomic sample in comparison to a reference-value made up of matched aneuploid samples. Furthermore, the test can be made more stringent by defining a smaller range.

An example of such a $\Delta\Delta C_T$ analysis, where trisomy 18 and 21 samples could be discriminated from each other as well as samples with normal ploidy for these chromosomes is illustrated as a scatter plot in Figure 4. This figure shows the clear segregation of the three different karyotypes. In addition, the manner by which the averaged ΔC_T -values of all relevant control samples can serve as calibrator or reference values are displayed in table 2. In our hands this simple procedure (and its

modifications as described in the methods section) has been shown to be most reliable, producing consistent results over several experiments and months.

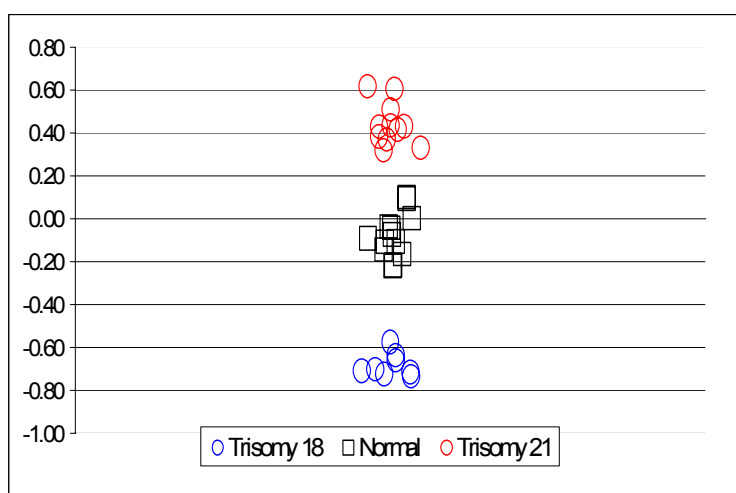


Figure 4. Scatter plot of control DNAs for a threshold of 0.45. Depicted are replicate averages per sample. The ΔC_T -values of all samples with normal karyotype group between -0.22 and $+0.10$, all trisomy samples lie outside of this area (trisomy 21 are above, trisomy 18 are below). The average ΔC_T -value of the normal samples is -0.09 . This value is used as the ΔC_T (calibrator) for samples with a normal karyotype at the threshold of 0.45 (refer to table 2).

1.3 Differences between the use of the SDS 7700 and SDS 7000 real-time PCR “Taqman” instruments

In our proof of principle study for the detection of trisomy 21 using real-time PCR we used the Applied Biosystems Sequence Detection System 7700 (SDS 7700) instrument which uses a complex laser-array for the excitation of the fluorescent-labelled reporter molecules. In this study, in which we only examined a small number of samples ($n=21$), we were able to correctly determine the karyotype in 10 of the 11 trisomy 21 samples analysed. The analysis also included 10 dilutions of the trisomy 21 samples. In two of the control samples and in 1 sample with trisomy 21 no definitive assessment was possible, due to poor quality or inadequate quantity of the DNA sample analysed.

In the interim period Applied Biosystems has introduced the smaller, less costly and user-friendlier SDS 7700 onto the market. This instrument obviates the need for a complex laser-based system, instead employing a halogen lamp for the excitation of fluorescent probes. After careful appraisal of this instrument, in which we determined

it to be as robust, reliable and as accurate as the more sophisticated and larger SDS 7000, we then adapted our assay in such a manner that it would permit the simultaneous analysis of trisomies 18 and 21.

This was achieved by replacing the chromosome 12 (GAPDH gene) control amplicon of the original trisomy 21 assay by a sequence on chromosome 18. In order to increase the specificity of our new assay, we also examined the influence of various other parameters such as PCR reaction mix composition, primer sequences and purity, as well as different probe chemistries. These studies were undertaken to ensure that the amplification efficiencies of the two reactions (chromosome 18 and 21) were equal.

As described above, reference calibration values for the two chromosomes being interrogated were determined by examining DNA samples with either normal, trisomy 18 or trisomy 21 karyotype (refer to table 2). Once we had established these reference calibration values, we next performed a large-scale study of almost 100 clinical amniotic fluid samples, in which we examined the ploidy for these two chromosomes in a blinded manner (*Zimmermann et al., manuscript in preparation*). In over 86% of the cases was a correct analysis regarding the ploidy for these two chromosomes possible. In those instances where we were not successful, the analysis was either hindered by inadequate concentrations of template DNA or by the presence of inhibitors in the original DNA preparation (**see note 7**).

These results clearly demonstrate the diagnostic potential of real-time PCR, whether for prenatal diagnosis or for other analyses involving gene-duplications or loss, such as are frequently found in cancer.

It is worth noting that although the Quantitative Fluorescent PCR analysis of STR has been established and clinically implemented for the rapid prenatal analyses of the most common aneuploidies, the use of real-time PCR does have a few advantages, in that it is more amenable to automation and, by being a closed system is less prone to contamination.

2. Materials

2.1 DNA extraction

1. For the extraction of DNA from the amniotic fluid samples, Chelex 100 resin (biotechnology grade 100 – 200 mesh sodium form) from Biorad (Reinach, Switzerland, catalogue number 142-1253) was used.
2. It is recommended to use a highly pure water source such as Analar water (molecular biology grade, VWR International). Alternatively Milli-Q water de-ionised with the Elgastat Maxima (Kleiner AG, Wohlen, Switzerland) can be used.

2.2 Performing real-time PCR

1. For our real-time PCR analysis we used an ABI PRISM 7000 Sequence Detection System (SDS7000) (**Note 1**).
2. For the PCR reactions we used MicroAmp Optical 96-well reaction plates (Applied Biosystems, Switzerland, Cat 4306737) and the ABI PRISM Optical adhesive covers (Cat 4311971) (**Note 2**).
3. The reactions were carried using the TaqMan Universal PCR master mix (Cat 4304437) containing Amplitaq Gold DNA Polymerase, AmpErase UNG, dNTPs with dUTP and passive Reference 1 (ROX fluorescent dye) (**Note 3**).
4. Primer and probe sequences are listed in Table 1 (**Note 4**).
5. The best results were obtained using TaqMan MGB probes (Cat 43160324) from ABI. These are 3'-labelled with a Minor Groove Binder (MGB) and a Non-Fluorescent quencher (**Note 5**). The Chromosome 18 probe is 5'-labelled with the fluorescent dye VIC, the chromosome 21 probe is 5'-labelled with FAM. It is advisable to store 5 µM aliquots at – 20 °C, where they are stable for over 1 year. If used rapidly (within 1 month), the probes can be stored at 4 °C. In this case, aliquots of both probes should be used and stored in parallel. Avoid exposure to light.
6. In our experience, the best results were obtained using HPLC purified primers (Microsynth, Switzerland) (**Note 6**). These can be stored as 10 µM aliquots at –20 °C. Primers stored at 7 °C are stable for several months.

Chromosome location	accession GenBank	Gene	Name	Fluorescent dyes 5'-3'	Sequence of primers	primer location bases
18p11.32	D00596	TYMS	Chr_18_F		TGACAACCAAACGTGTGTTCTG	2910 - 2931
			Chr_18_R		AGCAGCGACTTCTTTACCTTGATAA	2961 - 2985
			Chr_18_P_MGB	VIC-NFQ	GGTGTGTTTGGAGGAGTT	2933 - 2959
			Chr_18_P_dual	VIC-TAMRA	AAGGGTGTTTTGGAGGAGTTGCTGTGG	2930 - 2966
21q21.3	D87675	APP	Chr_21_F		CCCAGGAAGGAAGTCTGTACCC	7764 - 7785
			Chr_21_R		CCCTTGCTCATTGCGCTG	7826 - 7843
			Chr_21_P_MGB	FAM-NFQ	CTGGCTGAGCCATC	7788 - 7801
			Chr_21_P_dual	FAM-TAMRA	AGCCATCCTTCCCGGGCCTAGG	7795 - 7816

TYMS: Thymidylate Synthetase; APP: Amyloid Precursor Protein; NFQ: Non-FluorescentQuencher, MGB probe

Table 1: Primer and probe sequences for the real-time amplification of chromosomes 18 and 21. Probes used were the MGB probes Chr_18_P_MGB and Chr_21_P_MGB. The Sequences for dual labelled probes are included (see Note 5).

2.3 Analysis of real-time PCR data

1. No material required.

3. Methods

3.1 DNA extraction of amniotic fluid samples

1. Spin 1ml of amniotic fluid at 13,000 rpm in a bench top micro-centrifuge for 15 seconds to pellet the amniotic cells.
2. Discard the supernatant and re-suspend the cell pellet in 500 μ l of water.
3. Wash once by centrifuging the cells as in step 1. Discard the supernatant
4. Add 60 μ l of Chelex 100 resin to the pellet and re-suspend the cells by vortexing.
5. Incubate the samples for 20 minutes at 57 °C.
6. Following this, incubate the samples in a dry heat block at 100 °C for 8 minutes.
7. Next, prepare the sample for use in the real-time PCR analysis by heating to 95 °C for 5 minutes. Following this spin the samples at 13,000 rpm in a bench top micro-centrifuge for 2 minutes to pellet the resin. Carefully remove the aqueous DNA solution, taking care not to disturb the resin pellet (**Note 7**).
8. Use 2 μ l of this DNA solution in a final reaction volume of 25 μ l for the PCR analysis. (**Notes 8 and 9**).

3.2 Performing real-time PCR

1. It is advisable to include at least one control sample of known karyotype in the analytic run (**Note 10**).
2. Each sample should be analysed at least in triplicate (**Note 11**).
3. The preparation of the real-time PCR reactions should be carried out on ice.
4. The real-time PCR amplification is carried out in a total volume of 25 μ l (**Note 12**), containing 2 μ l of the sample DNA solution (**Note 13**), 300 nM of each primer (**Note 14**) and 200 nM of each probe (**Note 15**) at 1 x concentration of the Universal PCR reaction mix.
5. This is prepared by first pipetting the PCR reaction mixture into the reaction wells, following this add 2ml of the sample DNA solution (**Note 16**). Carefully seal the reaction plate with the optical adhesive cover. Centrifuge at 1400 rpm at 4 °C for 1 minute to spin down any droplets and remove air bubbles.

6. Immediately start the real-time PCR cycler with the emulation mode off (**Note 17**).
7. Following an initial incubation at 50 °C for 2 minutes to permit Amp Erase activity, 10 minutes at 95 °C for activation of AmpliTaq Gold and denaturation of the genomic DNA, use the following cycle conditions: 40 cycles of 1 minute at 60 °C and 15 seconds at 95 °C.

3.3 Analysis of real-time PCR data

8. Perform the experimental analysis with a baseline setting of 3 – 22 cycles (**Note 18**). Check the replicate curves for uniformity in the amplification plot view (**Note 19**) and for final fluorescence (**Note 20**).
9. Analyse 4 different thresholds at R_n -values of: 0.2 / 0.3 / 0.45 / 0.625, as indicated in Figure 2 and Table 2. (**Note 21**). Export the C_T result files and examine the data using an Excel spreadsheet.
10. In our experience, C_T -values from 25.0 to 29.0 (for a threshold of 0.2) will yield good results. C_T -values of higher than 29.0, are associated with inadequate quantities of DNA, which are too low to guarantee that preferential amplification of one of the multiplexed reactions does not take place (**Note 22**).
11. For every replicate calculate the ΔC_T for each of the four thresholds (refer to Table 2 and Figure 2).
12. For every replicate and for all three ploidies that can be distinguished, calculate the $\Delta\Delta C_T$ for each of the 4 thresholds:

$$\Delta\Delta C_{T \text{ calibrated}} = \Delta C_T (\text{sample}) - \Delta C_T (\text{calibrator})$$

13. Calculate for every replicate, with respect to a normal karyotype, to trisomy 18 and to trisomy 21, the average of the four $\Delta\Delta C_T$ -values.
14. The analysis of one replicate that gives the value closest to 0 for the averaged $\Delta\Delta C_T$ is indicative for the chromosomal status. This value should be smaller than ± 0.25 (**Note 23**), that is e.g.:

$$\Delta\Delta C_{T \text{ calibrated}} (\text{trisomy 18 sample}) = \Delta C_T - \Delta C_T (\text{calibrator trisomy 18}) = 0 \pm 0.25$$

15. If the results of all 3 replicates are indicative of the same chromosomal balance, the ploidy of the sample is that of the matched reference ΔC_T calibrators; otherwise the result is not conclusive but rather is suggestive (**Note 24**).

16. Alternatively it is possible to add the 4 calibrated $\Delta\Delta C_T$ -values. In this manner, a normal karyotype will have a value close to 0, whereas a trisomy 21 sample will be of the order of 2 and a trisomy 18 sample will have a value of approximately minus 2.

	ΔC_T calibrator values			
Threshold	0.2	0.3	0.45	0.675
Trisomy 18	-0.52	-0.55	-0.68	-0.73
Normal	0.01	-0.03	-0.09	-0.11
Trisomy 21	0.48	0.45	0.44	0.42

Table 2: The calibrator values for the three karyotypes and the four thresholds used. The average ΔC_T values of the control sample measurements for each karyotype are the respective calibrator values.

	Sample 1				Sample 2				Sample 3			
Threshold	0.2	0.3	0.45	0.675	0.2	0.3	0.45	0.675	0.2	0.3	0.45	0.675
C_T (chromosome 18)	27.83	28.47	29.25	30.17	27.91	28.47	29.17	29.96	27.21	27.81	28.45	29.28
C_T (chromosome 21)	28.07	28.66	29.34	30.15	28.53	29.24	30.00	31.01	26.65	27.28	28.01	28.80
ΔC_T	-0.24	-0.19	-0.09	0.02	-0.62	-0.77	-0.83	-1.05	0.56	0.53	0.44	0.48
$\Delta\Delta C_T$ (Normal)	-0.20	-0.10	0.06	0.25	-0.58	-0.68	-0.68	-0.82	0.60	0.62	0.59	0.71
$\Delta\Delta C_T$ (Trisomy 18)	0.30	0.40	0.61	0.79	-0.08	-0.18	-0.13	-0.28	1.10	1.12	1.14	1.25
$\Delta\Delta C_T$ (Trisomy 21)	-0.68	-0.57	-0.45	-0.30	-1.06	-1.15	-1.19	-1.37	0.12	0.15	0.08	0.16
Avg $\Delta\Delta C_T$ (Norm)	0.00				-0.69				0.63			
Avg $\Delta\Delta C_T$ (T 18)	0.53				-0.17				1.15			
Avg $\Delta\Delta C_T$ (T 21)	-0.50				-1.19				0.13			

Table 3: Experimental data and analysis of 3 samples (single replicates). The Average $\Delta\Delta C_T$ -values of the determined karyotypes are in bold. Sample 3 is one of the replicates depicted in figures 1 to 3. From this table it is apparent that sample 1 has a normal karyotype, sample 2 is of trisomy 18 and sample 3 is trisomy 21 (it is one of the replicates depicted in figures 1 to 3).

4. Notes

1. In order for this assay to function, the main requirement for the real-time PCR instrument is that the fluorescence data is normalised to the passive reference dye, such as the ROX dye used by ABI. Any instrument that offers this feature should be suited for the test. In a previous study we used the ABI PRISM 7700 Sequence Detection System successfully.

2. The optical covers have a high light transmission capacity, a necessity for highly precise measurements. Although optical caps should also work (according to the manufacturers' technical support), our experience shows that the automatic data collection adjustments of the SDS7000 do not suffice to achieve the accuracy needed. For the laser illuminated SDS7700 the covers and caps work fine. However, it is advisable to always use the same covering for the reaction wells, since it has an influence on the measured fluorescence.

3. The use of AmpErase UNG is absolutely required to avoid amplification of any miss-primed non-specific products formed prior to specific amplification. If this step is omitted, the balance of the two multiplexed reactions can be lost and one of the sequences will be amplified with a greater efficiency, resulting in false results. For the same reason use of a Taq- Polymerase activated by a hot start is required.

4. To match the two assays a variety of primer combinations for the chromosome 21 were tested. For the sake of expedience we have listed only the most efficient ones as well as the pertinent MGB probes (refer to Table 1).

5. MGB probes emit lower background fluorescence than dual labelled probes. This is mainly due to more efficient quenching because the probes are shorter. As a result, MGB probes have a longer observable exponential phase, and this permits the use of a wider range for the thresholds. Dual labelled probes may also be used, but the measurements are slightly inferior in terms of final accuracy.

It is also important to be aware, that the use of different batches of the same probes can result in differences in measurement due to minor differences in quality.

6. It is highly recommended to use HPLC purified primers, as these are not subject to artefacts that non-purified primers are prone to, such as the formation of unspecific products by the presence of shorter unspecific primer fragments, which have an adverse effect on the reaction efficiency. Non-purified primers are also subject to greater batch-to-batch variation than HPLC purified ones.

7. It is very important that the Chelex resin suspension is heated prior to centrifugation and separation of the DNA from the resin pellet. If not, re-adsorption of the DNA by the resin can occur, thereby removing a major amount of the DNA from the solution. This adsorption process may also lead to an imbalance of the ratio of the two target chromosomes. It is also important that no resin is transferred to the PCR as this will adversely affect amplification.

8. DNA concentrations need not to be quantified. The Amount of DNA needed for highly reproducible results ranges from 5 ng to 80 ng. The protocol will usually produce a DNA solution in the required concentration range. Because of the limited quantity of sample material available, it should not be used for concentration measurements. The additional working step is not necessary.

By co-amplifying a reference sample of known amount of DNA, which is the usual practice, the concentration of the sample can be determined with the following equation:

$$\text{concentration of sample} = 2^{-(\Delta CT)} / \text{concentration reference sample}$$

9. Any kind of genomic DNA where the chromosomal ratio is preserved can be used. Next to the chelex extracted amniotic fluids we also tested extractions with the high pure PCR template kit from Roche and with the QIAmp blood kits from Qiagen. Cellular materials used were whole blood, monocytes from blood, cultured cells from blood and from amniotic fluid.

10. Use of a reference sample of known quantity allows quantification as mentioned in Note 8. If the sample and a dilution of it are amplified, the sequence detection

software can determine the concentration of all samples tested with two standard curves (one for each chromosome).

In theory these curves would permit karyotyping of the samples, however the results are not good enough.

11. It makes sense to amplify a sample and a dilution of it. Sometimes in the original sample PCR inhibitors are present, that are diluted with the DNA. This added precaution makes it possible to observe reaction efficiencies for a sample via a standard curve and thus reveal the presence of inhibitors.

12. Reaction volumes tested were 20 μ l to 50 μ l. Using 20 μ l works, but the deviations are increased. A volume of 50 μ l of course works fine, but it is not at all necessary to use such a big volume. Very small differences in collected data might occur, if the reaction volume is different from the volume that the assay is characterised for.

13. If the concentration of the DNA is too low, up to 4 μ l chelex extracted solution can be used. If the concentration is still too low, DNA has to be up-concentrated by for example evaporation, precipitation or extraction into a smaller volume using a kit. To save DNA and for highly concentrated samples, smaller volumes or dilutions in water can be used.

14. Higher primer concentrations result in a decrease of the reaction efficiency in the multiplex assay. This can be attributed to an increase of unspecific reactions. Also higher concentrations of genomic DNA disturb the reaction.

15. Probe concentrations of 100 μ M also work fine, but a little accuracy may be lost, especially at the threshold of 0.675.

16. Sample set-up on ice is important to reduce non-specific product formation to a minimum. The reaction plate should be kept on ice at all times prior to starting the real-time PCR.

17. Run the program always at the same heating and cooling rates. Differences in the temperature program will result in slight differences of the final C_T and ΔC_T -values. We use the emulation off setting, because it is faster.

18. The baseline setting is very important to achieve optimal results in the analysis. It is used to separate unspecific fluorescent signal from the signal generated by the reaction. If the baseline is too far away from the amplification curve, many results of especially the lower thresholds will be affected by noise.

Setting the baseline too close will skew the amplification curves and change the ΔC_T to smaller values.

19. In the amplification plot view check if the amplification curves of both dyes look normal and parallel for all three replicates. If the three replicates do not look very similar something might be wrong with at least one of the reactions. This can be due to contamination with DNA (pipetting errors), dirt or fluorescence source other than the dyes, leakage of the well, presence of an inhibitor or other reasons. If the curves do not resemble each other closely, the test should be re-run.

20. After 40 cycles of amplification the ΔR_n will usually be between 2.0 and 3.0. Data from runs with a final ΔR_n of less than 1.5 is of poor quality.

If using dual labelled probes, the ΔR_n will be smaller than when using MGB probes. This is a result of less efficient quenching and resulting higher background fluorescence in the longer dual labelled probes.

21. The use of several thresholds increases the accuracy of the method. By using the four proposed thresholds, fluorescence data collected during 3 or 4 cycles is used compared to data from only 2 cycles when using only 1 threshold. A threshold of 0.1 sometimes contains a good amount of unspecific signal. To exclude that possibility, the minimal threshold setting used is 0.2. The maximum threshold of 0.675 still gives good data, although the reaction is at the end of the exponential phase and efficiencies decreasing.

22. At DNA concentrations that result in a C_T of 30.0 or higher (at the threshold of 0.2), deviations occur in the amplifications, sometimes resulting in ΔC_T -values uncharacteristic for the sample karyotype. Using a cut-off of 29.0 is a security measure to guarantee balanced amplification of both targets.

The cut-off of 25.0 is a result of the baseline setting. Also the amplification of genomic DNA of very high concentrations can result in lower and varying efficiencies. None of the 100 amniotic fluid samples tested had a high enough concentration to result in such a low C_T .

23. To make the test more stringent, the maximum average $\Delta\Delta C_T$ -value can be set to a smaller value. Alternative stringency requirements can be to demand that the results of a minimal number of thresholds per sample are indicative of the normal karyotype or one of the trisomies (e.g. 10 of total 12 values per triplicate).

24. It is usually possible to exclude one of the three karyotypes.

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Large-Scale Real-Time Quantitative PCR Analysis for the Detection of Trisomies 18 and 21.

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Chapter 7

Abstract

Objective: We report the results of the first large scale study using a novel real-time quantitative PCR (real-time qPCR) method for the detection of fetal trisomies. The multiplex real-time TaqMan assay was performed on 97 clinical amniotic fluid samples which were analysed in a blinded manner. Results were compared to those obtained by quantitative fluorescent PCR (QF-PCR) of short tandem repeats (STRs) in a diagnostic laboratory. The aim of this study was to evaluate the diagnostic potential of the new real-time qPCR method.

Results: The ratio between the copy numbers of chromosomes 18 and 21 was assessed by multiplex real-time qPCR. In our analysis, eight samples had to be excluded as the sample volume was too small. Our analysis of the remaining 89 samples resulted in a specificity of 94%. The five false diagnoses were due to incorrect sample handling. Upon identification of the source of error, this was readily addressed leading to a correct re-examination of these samples.

Our data, therefore, demonstrate that, with certain precautions, the new real-time qPCR assay allows the determination of fetal aneuploidies with 100% specificity.

Conclusion: The real-time qPCR is a rapid and straightforward method for the detection of prenatal aneuploidies and is highly amenable to automation at comparable costs and sample requirements as conventional QF-PCR.

Introduction

Prenatal diagnosis of chromosomal abnormalities is currently accomplished by invasive techniques, such as amniocentesis and chorionic villus sampling. Following these sampling methods a complete karyotype is established, a procedure that takes on average two weeks for cell culture. This period of considerable parental anxiety led to the need for faster methods. Newer laboratory techniques such as Fluorescent In-Situ Hybridisation (FISH) and QF-PCR of STRs (Mansfield 1993); (Pertl et al. 1999), (Levett, Liddle, and Meredith 2001) can be used to obtain a rapid preliminary diagnostic answer within 24 – 48 hours. To date, these methods complement but do not replace full karyotypic analysis as they only provide an answer for a limited number of numerical aberrations (13, 18, 21, X and Y), which cover about 70% of all pertinent fetal chromosomal aberrations (Evans et al. 1999). The ability to rapidly determine major chromosomal abnormalities in less than two days after the invasive procedure offers the possibility of quick intervention in case of adverse results. Recent studies have indicated that of the two approaches the FISH method is expensive and labour intensive (Eiben et al.), while QF-PCR is more suited for high throughput analysis. However, since special technical expertise and equipment are needed, QF-PCR remains confined to laboratories with methodological experience and with access to specialised software for the data analysis (Hulten, Dhanjal, and Pertl 2003).

In the past decade, real-time qPCR, employed as the 5'-nuclease TaqMan assay, has emerged as a very powerful tool for the measurement of the copy number of nucleic acid sequences. The method is more sensitive, specific and reproducible than previous approaches. It has consequently found a wide spread application in the analysis of gene expression, determination of gene deletions or duplications in cancer research as well as in the quantitation of circulating nucleic acids in body fluids for prenatal, viral and cancer diagnosis and prognosis. Newly developed instruments permit certain assays to be performed within 15 minutes.

Although manufacturers generally state that current instrumentations allow only the determination of 2-fold differences in template concentration, we have recently shown, that multiplex real-time qPCR assays can be optimised to

detect as low as 50% differences in template copy number. This is a prerequisite when attempting to determine fetal aneuploidies (Zimmermann et al. 2002). This increased accuracy was achieved by implementing special experimental and analytical parameters.

We have now extended upon this preliminary investigation by developing the assay further, permitting the simultaneous assessment of chromosomes 18 and 21, and have tested the diagnostic potential of the assay in a blinded large-scale retrospective study of clinical samples. Our results indicate that real-time qPCR is a powerful tool for the prenatal diagnosis of aneuploidies.

Material and Methods

Clear amniotic fluid samples from 97 pregnant women were collected and DNA extracted and analysed by QF-PCR for STRs at the Cytogenetic DNA Services Ltd., London (Levett, Liddle, and Meredith 2001). Small aliquots of the archived DNA samples were coded and sent to Basel for blinded real-time qPCR analysis.

Washed cells from 1ml of amniotic fluid were suspended in 60 µl of a 10% suspension of chelex 100 resin in water (biotechnology grade 100 – 200 mesh sodium form, Biorad, UK) and incubated for 20 minutes at 57°C, then for 8 minutes at 100°C. The resin was pelleted at 13000 rpm for 2 minutes and the aqueous DNA solution used for PCR analysis.

For control purposes, DNA was extracted from 400 µl amniocytes cultures using the QIAmp DNA Blood Mini Kit according to the manufacturer's recommendations (Qiagen, Switzerland). In addition, already diagnosed samples were re-examined.

Karyotype determination by real-time qPCR

The multiplex real-time qPCR analysis for sequences on chromosomes 18 and 21 was performed in the ABI PRISM 7000 Sequence Detection System (Applied Biosystems (ABI), Switzerland). Primers and TaqMan MGB probes were designed to amplify a sequence of the amyloid precursor protein gene in the Down's region of chromosome 21 and of the thymidylate synthetase gene on chromosome 18. Sequences for chromosome 21 were: Primers 5'-CCC AGG AAG GAA GTC TGT ACC C-3' and 5'-CCC TTG CTC ATT GC GCT G-3', and probe, 5'-CTG GCT GAG CCA TC-3', with Minor Groove Binder (MGB) and labelled with FAM fluorescent dye. Chromosome 18 sequences for primers 5'-TGA CAA CCA AAC GTG TGT TCT G-3' and 5'-AGC AGC GAC TTC TTT ACC TTG ATA A-3', and the VIC-labelled MGB probe 5'-GGT GTT TTG GAG GAG TT-3'.

Patient DNA in volumes of 2 µl was amplified in triplicate in 25 µl reactions containing 300 nM of each primer (HPLC purified, Microsynth, Switzerland), 200 nM of each MGB probe (ABI), and 1 x concentration of the TaqMan

Universal PCR master mix (ABI). The reactions were prepared on ice and cycling conditions were as follows: 2 min at 50°C and 10 min at 95°C for initial denaturation of the genomic DNA and polymerase activation, followed by 40 cycles of 1 min at 60°C and 15 s at 95°C.

The real-time qPCR analysis was performed with a baseline setting of 3 – 22 cycles. Amplifications with a final normalised fluorescence (ΔR_n) smaller than 1.50 were excluded from the karyotype determination due to insufficient amounts or quality of DNA.

Each reaction curve was analysed at 4 different thresholds at ΔR_n -values of: 0.2 / 0.3 / 0.45 / 0.625, with an increase in fluorescence intensity of 1.5 fold between thresholds. Amplifications with C_T -values from 25.0 to 29.0 (for a threshold of 0.2), corresponding to approximately 700 – 10000 genome equivalents per PCR, were valid for diagnosis. Samples with values outside of this range were re-tested either in dilution or with a larger sample input volume.

For the three replicates the ΔC_T and the $\Delta\Delta C_T$ for each of the four thresholds was determined, leading to 12 single $\Delta\Delta C_T$ values per sample:

$$\Delta C_T = C_T (\text{Chromosome 18}) - C_T (\text{Chromosome 21})$$

$$\Delta\Delta C_{T,\text{calibrated}} = \Delta C_T (\text{sample}) - \Delta C_T (\text{calibrator})$$

The ΔC_T (calibrator) is the average ΔC_T of the reference samples of normal karyotype. In this analysis, we averaged the ΔC_T -values of all valid reference DNAs.

For the determination of a sample karyotype the following criteria had to be met:

1. At least nine of the twelve single $\Delta\Delta C_{T,\text{calibrated}}$ values had to be in the range from -0.25 to +0.25.
2. The averaged $\Delta\Delta C_T$ -values of each of the three replicates had to be in the range from -0.25 to +0.25.

If the sample karyotype was not normal, the same calculations were performed with ΔC_T (calibrator) values derived from the trisomy controls. If the results were not indicative of any karyotype, the sample was retested.

Results

Our analysis indicated that for a reliable assay the sample input needs to be within a certain concentration range. We found that between 700 and 10000 GE are optimal for diagnosis. Most of our samples contained this amount in 2 μ l, the usual input volume. Amplification of standard samples in fourfold dilutions in this range showed equal and optimal efficiencies, indicating accurate and reproducible assay performance [figure 1].

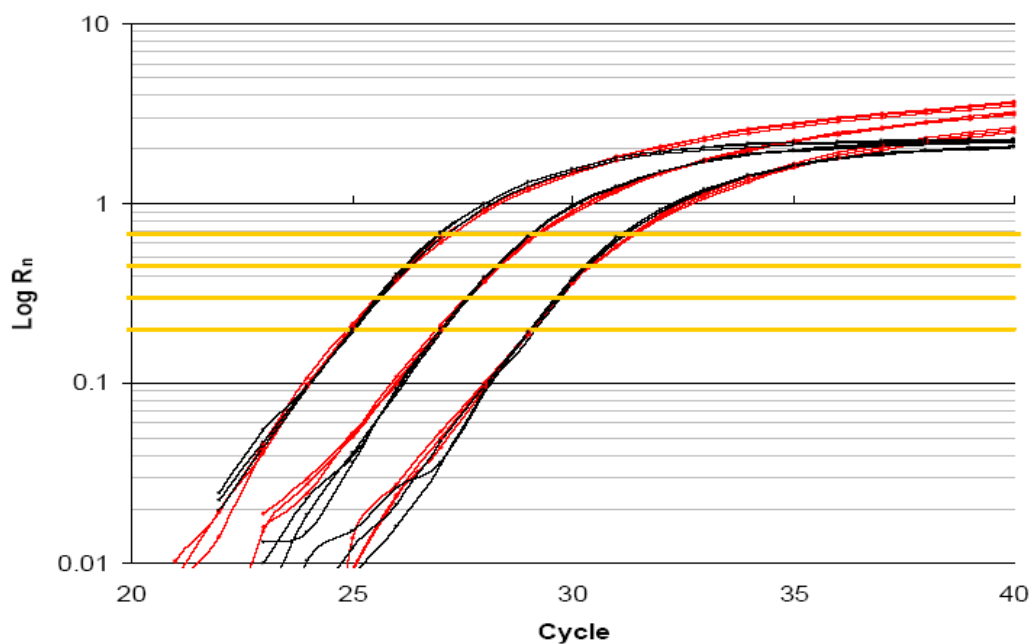


Figure 1: Amplification plot of the real-time PCR data. Triplicate amplifications of a normal karyotype sample in fourfold dilution. The red curves represent data from chromosome 21, the blue curves from chromosome 18. The four thresholds used in analysis are represented by yellow lines. The efficiencies determined for chromosome 18 were between 1.95 and 1.98 for the four selected thresholds, for chromosome 21 between 1.93 and 1.96.

Analysis of standard samples, which included cases of trisomy 21 and 18, confirmed that the ΔC_T -values of the amplified samples cluster in three specific groups depending on their karyotype [figure 2]. By defining a cut-off region of ± 0.25 cycles around the ΔC_T (calibrator) of each ploidy, the ΔC_T of a sample allows to assess its karyotype. The ΔC_T -values were stable over several experiments, as is evident from the analysis of normal samples in 9 consecutive experiments [Figure 3].

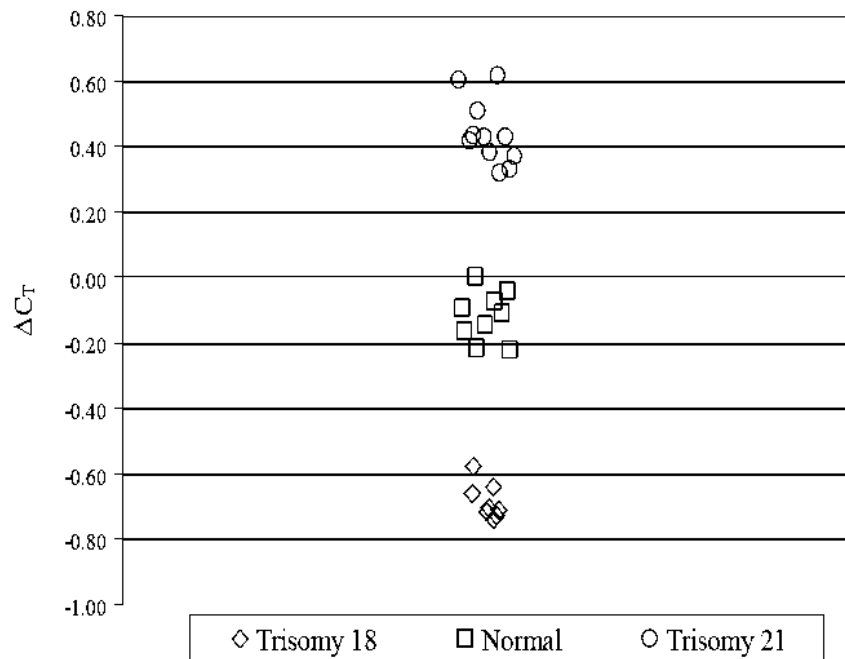


Figure 2: Scatter plot of the ΔC_T -values of the control DNA samples for the threshold of 0.45. Depicted are replicate averages per sample. The ΔC_T -values of all samples with normal karyotype group between -0.22 and +0.10, all trisomic samples lie outside of this area. The average of the normal samples is -0.12, this is the ΔC_T (calibrator) for normal karyotype at threshold 0.45.

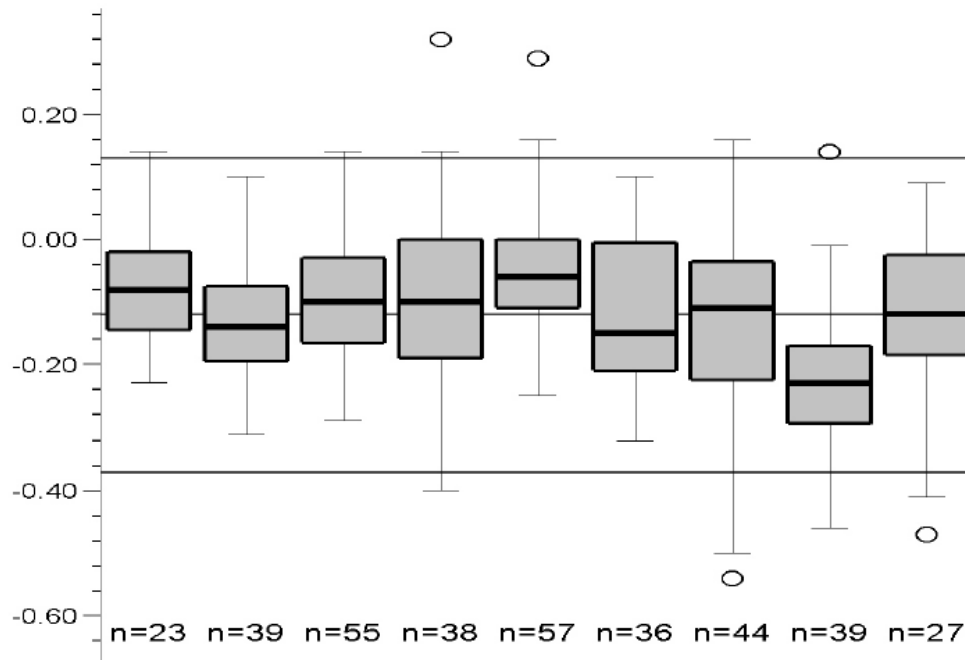


Figure 3: Box plot of C_T -values in 9 experiments of all samples of normal karyotype at the threshold 0.45. Numbers on X-axis indicate number of measurements per experiment. The cut-off lines for normal karyotype are at -0.37 and 0.13, the median line at -0.12. A few points are outside of the cut-offs, but replicate amplifications and values from other thresholds prevent misdiagnosis.

In our study we examined 97 samples, of which 8 had to be excluded due to too small sample volume. Our blinded analysis of the remaining 89 samples indicated that the method could be reliably used for the analysis of fetal aneuploidies, as we were able to correctly diagnose all 11 cases with trisomy 21 and all 4 with trisomy 18. 68 samples of normal and one of triploid karyotype were correctly determined as chromosomal balanced. It is an intrinsic feature of this real-time qPCR assay that it cannot distinguish triploid from normal karyotype samples, as the chromosome ratios are equal. In our blinded study, 5 samples with a normal karyotype were misdiagnosed.

The analysis of aneuploid samples indicated that these could be detected with a sensitivity of 100% (15/15), and a false negative rate of 0%. In contrast, our study showed that the false positive rate of 25% (5/20) was very high. As we repeatedly observed erroneous results from these samples, we hypothesised that this error may be due to an artefact introduced during sample processing, such as aberrant adsorption to the resin used in the DNA purification procedure. We re-extracted the DNA from the resin of three such previously misdiagnosed samples by addition of 40 µl water and heating to 95 °C for 5 minutes. The reanalysis showed that the fetal ploidy could subsequently be correctly determined [figure 4]. Unfortunately we could not re-examine the other two samples which had yielded erroneous results, due to insufficient amounts of material remained for reanalysis. We conclude, that the error was introduced during sample handling. Following purification with conventional resin based technology, storage and shipment, the recovery of DNA from these samples was poor. This source of error could be excluded by re-solubilizing the DNA under heat. The improved recovery of DNA was also observed in samples with initially insufficient amounts of DNA.

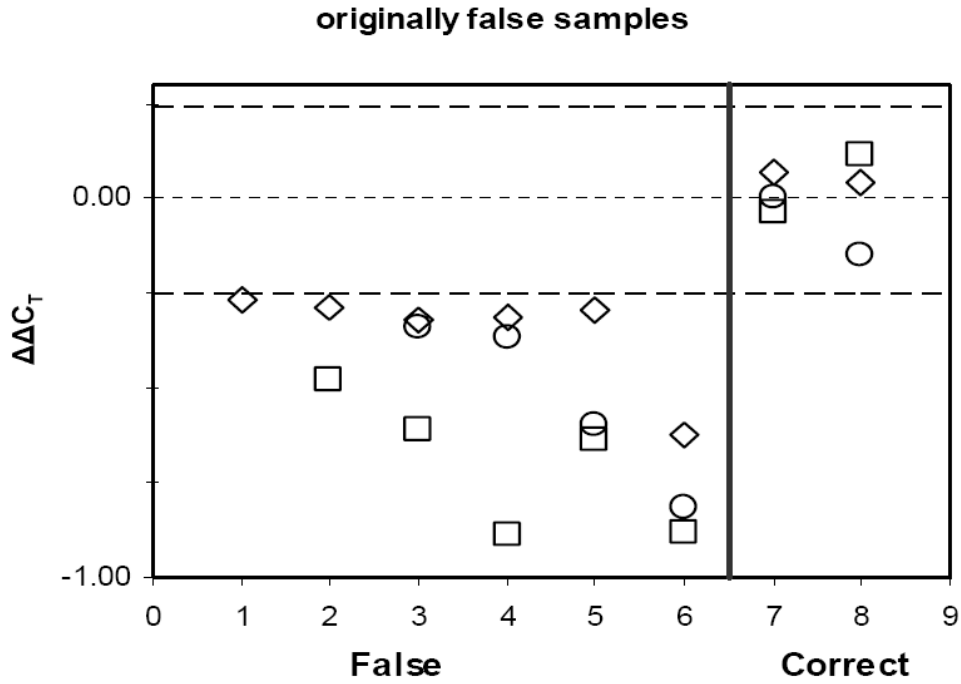


Figure 4: The corrected C_T -values of 3 false samples. Experiments 7 and 8 are the results from the re-solubilized resins. C_T -values between -0.25 and 0.25 indicate that the normal karyotype is determined correctly. $C_T = C_T(\text{sample}) - C_T(\text{calibrator})$.

The real-time qPCR assay *per se* is highly robust, but it needs sufficiently concentrated and pure DNA. Further indication is presented by the measurements of the control samples: These DNA solutions were extracted to high purity by silica membrane technology and optimally suited for the real-time qPCR assay. Resulting deviations of the ΔC_T -values are clearly smaller than for the clinical samples: we plotted the ΔC_T against the ratio of the chromosomes and fitted an exponential curve. Excluding the five false samples, all replicates of the study give R^2 values of 0.74 to 0.80, depending on the threshold. Plotting the values of all reference samples results in R^2 values of 0.89 to 0.95.

In conclusion, using only 1 assay, we were able to determine the karyotype in 84 of 89 fetal samples, with a combined false negative rate of 0% and a false positive rate of 25% for both trisomies. The specificity of 94% (84/89) underestimates the accuracy of the method considering the finding of DNA adsorption by the extraction resin. Upon analysis of re-solubilized DNA we improved the specificity to 100%.

Ratio determination

The use of a ΔC_T - or $\Delta\Delta C_T$ -value is not demonstrative for the diagnostic application, as the analyst should be supplied with a value concerning the actual chromosomal copy numbers. For this reason, converting the amplification data into a ratio of chromosome copy numbers is desirable, such that the diagnostic answer of the assay will be easy to understand and output values linear (Livak and Schmittgen 2001). A proviso for the ratio determination by the $\Delta\Delta C_T$ -method is that both targets are amplified with equally optimal efficiencies. We showed earlier that this condition is met, consequently the ratio between the two chromosomes can be calculated by the following formula:

$$\text{Chr21/Chr18} = 2^{-(\Delta\Delta C_T)} = 2^{(C_{T,\text{sample}} - C_{T,\text{calibrator}})}$$

We applied the averaged $\Delta\Delta C_T$ values of all measurements as calibrator-values. The resultant ratios for normal karyotype samples are averaging 1.00 at every threshold [table 1]. The ratios calculated for the trisomy samples, however, are closer to 1.00 than would be expected. Consequently, when using the values of the calculated ratios for diagnosis, one needs to adjust the cut-off values for this bias.

Sample karyotypes	Replicates	ΔC_T (threshold 0.2)	ΔC_T (threshold 0.3)	ΔC_T (threshold 0.45)	ΔC_T (threshold 0.675)	Average $\Delta\Delta C_T$	Average Ratio
Trisomy 18	62	-0.53	-0.59	-0.68	-0.78	-0.55	0.69
balanced	358	-0.01	-0.06	-0.12	-0.19	0.00	1.00
Trisomy 21	103	0.45	0.40	0.37	0.33	0.48	1.40

Table 1: Average ΔC_T values of all measurements separated by karyotype. The average $\Delta\Delta C_T$ values are calculated in comparison to the balanced samples' ΔC_T values. These values are used for the ratio determination.

Discussion

In the past decade, QF-PCR of STRs has emerged as the prime PCR method used for the rapid detection of chromosomal aneuploidies. Recently we presented an alternative PCR approach for the detection of trisomy 21 (Zimmermann et al. 2002), which is based on real-time PCR. In this assay, a sequence in the Down's syndrome critical region of chromosome 21 and a control sequence from the GAPDH gene on chromosome 12 are simultaneously amplified. Our analysis indicated that trisomy 21 samples could be reliably distinguished from karyotypically normal specimen according to the resulting ΔC_T . This report represented the first instance that real-time qPCR could be used with a sensitivity that demonstrated a 3:2 difference between gene loci (Armour et al. 2002). Also other PCR methods have been presented to avoid the use of polymorphic sites, which is encumbered with small disadvantages that restrict its application to experienced laboratories (Mann et al. 2001). The suggested alternatives involve the QF-PCR comparative quantification of non-polymorphic loci (Lee et al. 1997); (Rahil et al. 2002), allele quantification by melting curve analysis of SNPs (single nucleotide polymorphisms) (Pont-Kingdon G. and Lyon 2003) and multiplex ligation dependent probe amplification (MLPA) (Slater et al. 2003).

We modified our initial assay to quantify chromosome 21 in relation to chromosome 18 and thus test for two of the most prevalent fetal aneuploidies. The advantage of the multiplex PCR set-up is that it circumvents problems associated with well to well variations between the amplification targets caused by reaction set-up, by the thermocycler or by the changing conditions during the reaction (Li et al. 2003).

Assay validation

The major preconditions to achieve the highest degree of reproducibility and precision are stable and optimal amplification efficiencies (Tichopad, Dzidic, and Pfaffl 2003). In the present study we showed equal efficiencies for both targets amplified in multiplex over a wide range of concentrations and fluorescence intensities. This was ascertained by a series of standard dilution curves.

Our studies indicate that the following specifications are critical for optimal performance: purified primers are an absolute prerequisite for high accuracy in PCR, while being a negligible cost factor. Also, the properties of the TaqMan MGB probes allow very accurate fluorescence measurements. The MGB probes generate a low background fluorescence, which results in a long observable exponential phase of the PCR reactions. This is especially important in multiplex experiments, where the total fluorescence is increased by the presence of more than one labelled probe.

In order to level out small fluctuations and to ensure equal amplification of both sequences in each analysis, 4 thresholds along the linear phase of the amplification plot are chosen for the ploidy determination. This approach is more accurate than the use of a single threshold, which can be vitiated by background fluorescence at low thresholds and plateau effects at high thresholds, in addition to the common fluctuations in the fluorescence measurements.

The use of a single sample as reference is not reliable and may result in an erroneous diagnostic result. This caveat can be overcome by basing the calculations on the control measurements of several experiments. This procedure increases the accuracy of the ΔC_T (calibrator) value. As long as identical reaction conditions are ensured, the generated data is very stable and reproducible (Rutledge and Cote 2003).

PCR bias

We observed that the multiplex amplification suffers from ΔC_T -shifting when the targets are present in similar concentrations. This bias against the more abundant locus towards the PCR product ratio of 1:1 was already observed in our previous pilot study (data not published) and has been reported for the later PCR cycles (Suzuki and Giovannoni 1996; Mathieu-Daude et al. 1996). Data from the recently reported MLPA describes an even more decreased, non-linear relationship between chromosome dosage and the mean peak ratios of PCR products with unclear cause. We suggest that the rate of amplification of the more abundant PCR product declines faster than that of the less abundant one because of the self-hybridisation of PCR products, thus preventing primer annealing to a fraction of the template. The higher self-inhibition during all

PCR cycles for the more abundant product results in a successive decrease of the original difference.

Aneuploidy detection by real-time qPCR

Our findings demonstrate that real-time qPCR can be a fast and reliable method for the diagnosis of the major chromosomal abnormalities. During this study, we were alerted to pitfalls of PCR-based methods. Main concerns in the clinical application are DNA quality and quantity. DNA adsorption by the extraction resin led to erroneous diagnosis of five samples. This can be prevented by using freshly extracted DNA, resolubilising stored samples, or by using a different DNA extraction method.

Our study also indicated that a minimum template concentration in the reaction mixture is required to ensure equal amplification. The amount necessary is usually present in 1 ml of amniotic fluid and comparable to the requirement for QF-PCR based assays. Advantages of the real-time method in this regard are that the assay is valid for concentrations ranging over more than one order of magnitude and that the C_T -value of the amplification directly indicates insufficient template quantity. As such, the accurate determination of template concentration is possible without additional work or sample demand.

It should be kept in mind, that the precision of the PCR for high copy numbers is better, as low amounts of DNA cause a higher degree of variability. Preferential amplification (Findlay et al. 1998) and statistical variation due to sampling become important impediments (Niesters 2001). We observed that 700 GE per reaction are sufficient to assure optimal assay performance. However, the lower limit of sample material was not explored extensively, but good sample quality and smaller reaction volumes can improve the assay performance further.

Several quality control measures are integrated in the real-time qPCR set-up and permit monitoring of the efficacy of the assay. These are available without any additional hands-on time, and include the final fluorescence (R_n), the slopes of the amplification curves and uniformity of replicates. Abnormalities in these parameters allow to identify samples that are inadequate to guarantee accurate assay performance.

The absence of post-PCR processing saves substantial time and the closed tube system effectively eliminates the risk of carry-over contamination, a vitally

important factor for the clinical setting. This consequently reduces the demand on space and equipment, as separate locations for PCR product handling are not necessary.

Akin to other PCR based methods, real-time qPCR is a suitable tool for the diagnosis of the most common chromosomal aberrations, in our case specifically for the aneuploidies of the chromosomes 21 and 18. The assay can be readily extended for the analysis of the other common fetal aneuploidies of chromosomes 13, X and Y. One shortcoming of the method is that triploidies cannot be detected, as these result in an analysis indicating chromosomal balance. It will only be possible to detect these if one or two Y chromosomes are present, that is 69XXY or 69XYY. As triploidy is almost always ascertained by ultrasound examination, this is not a major concern.

To date, the real-time qPCR method is limited in the number of loci that can be analysed in one well, in that only five different fluorophores can be monitored simultaneously. Other methods exhibit a much greater multiplexing capacity, for example QF-PCR of STRs allows to target up to 12 loci and MLPA even 40 in one reaction. On the other hand, in the real-time PCR assay only one locus per chromosome needs to be examined. Additionally, the system and data analysis are very amenable to automation. Currently a 384 well format is available requiring as little as 5 – 10 µl reaction volume, which allows a very cost-effective and flexible throughput.

A further advantage is that the real-time qPCR is the fastest assay reported to date. With rapid cycling, analysis times shorter than half an hour are easily possible, and an adapted software could make data analysis almost instantaneous. By automating DNA extraction and the subsequent amplification, the real-time qPCR test can be conducted in as much as no laboratory training is necessary to conduct the test. This potential to obtain a diagnostic result within a few minutes at the site of the invasive sampling procedure cannot be matched by other methods.

In addition, it is possible to combine the assay with ancillary methods for the detection of aneuploidies, such as SNP based melting curves and even QF-PCR. As there is a trend to use molecular methods as stand alone tests for women who are at relatively low risk for having a baby with a chromosome disorder (Hulten, Dhanjal, and Pertl 2003), it seems advisable to combine

methods that can be carried out simultaneously to complement individual advantages.

In summary, we report the first large study using real-time qPCR for detection of fetal aneuploidies. The accuracy and precision of the real-time technique is clearly evident. Our study also indicates that the method is straightforward and that it can produce results of diagnostic value within as little as two hours.

With further development of the instrumentation and reagents the test can be easily optimised to a high-throughput method, which would save time and money and would allow to analyse an increased number of targets.

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Size Separation of Circulatory DNA in Maternal Plasma Permits ready Detection of Fetal DNA Polymorphisms.

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Chapter 8

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ABSTRACT

Background: Circulatory fetal DNA in maternal plasma has rapidly been introduced as a new method for non-invasive prenatal diagnosis, particularly for the analysis of fetal genetic traits, which are absent from the maternal genome, e.g. RHD or Y chromosome specific sequences. Currently, the analysis of other fetal genetic traits has proven to be more problematic, due to the overwhelming presence of maternal circulatory DNA sequences. We have examined whether different biochemical properties can be discerned between fetal or maternal circulatory DNA.

Methods: Circulatory plasma DNA was examined by agarose gel electrophoresis. The fraction of fetal and maternal DNA in size fractionated fragments was determined by real-time PCR. The determination of paternally and maternally inherited fetal genetic traits was examined using highly polymorphic chromosome 21 specific microsatellite markers.

Results: Size fractionation of circulatory DNA indicated that the major portion of cell free fetal DNA had an approximate molecular size of less than 0.3 kb, whereas maternally derived sequences were on average considerably larger than 1 kb. Analysis of size fractionated DNA (≤ 0.3 kb) from maternal plasma samples facilitated the more ready detection of paternally and maternally inherited microsatellite markers.

Conclusions: Circulatory fetal DNA can be enriched for by size selection of fragment sizes of less than approximately 0.3kb. Such selection permits more ready analysis of both paternally and maternally inherited DNA polymorphisms.
(222 words)

INTRODUCTION

Since its discovery in 1997 by Lo and colleagues (1), circulatory fetal DNA in maternal plasma or serum has rapidly emerged as the prime strategy for the development of risk free methods for prenatal diagnosis of fetal genetic traits (2, 3). Indeed, due to the relative abundance of this fetal genetic material, which is several orders of magnitude higher than that of trafficking fetal cells (4), the determination of fetal genetic loci which are totally absent from the maternal genome has proven to be relatively facile. Consequently the analysis of circulatory fetal DNA in maternal plasma is already being offered clinically by several centres for the determination of the fetal RHD status in pregnancies with a Rhesus constellation, or the determination of fetal sex, via the detection of Y chromosome specific sequences in pregnancies at risk for an X-linked disorder (e.g. Haemophilia, Fragile X syndrome) (3).

Quantitative analysis of this new found fetal analyte using real-time PCR strategies have also indicated that the concentration of circulatory fetal DNA were elevated under a variety of pregnancy related pathologies including preeclampsia (5, 6), preterm labour (7, 8), *hypermesis gravidarum* (8) and in pregnancies with fetal aneuploidies, most notably trisomy 21 (9, 10). These studies have suggested that fetal DNA concentrations may serve as a new screening marker for such pregnancy related anomalies (11).

A caveat of current investigations is that the overwhelming amount of circulatory DNA in the maternal circulation is of maternal origin (>90%) (4), which has rendered the discrimination of more subtle genetic differences between mother and child considerably more difficult (2, 3). This is generally true for Mendelian genetic disorders involving point mutations (12), or those instances where both parents are carriers for the same disease allele, as well as for the examination of DNA polymorphisms (13, 14), which could be used for the determination of fetal ploidy (15). Consequently few reports exist regarding the successful use of circulatory fetal DNA for such applications (12, 16-18).

To date few studies have addressed the biochemical properties of circulatory fetal DNA (19, 20). In the most recent of these Chan and colleagues (20) used differently sized PCR amplicons to discern the respective size distributions

of circulatory fetal and maternal DNA species, which indicated that fetal DNA molecules are generally smaller than comparable maternal ones. As this approach only permitted a precise delineation of rather small DNA species (<800bp), we have examined this facet using a combination of agarose gel electrophoresis, Southern blotting and real-time PCR, as this would permit an analysis of large DNA molecules (> 10 to 20 kb). Our studies have shown that circulatory DNA has apoptotic characteristics, displaying a typical ladder obtained by nucleosomal cleavage. We also observed that circulatory fetal DNA is generally of a smaller size than maternally derived cell free DNA fragments, a feature which is in good agreement with recent observations (20). By exploiting this observation, we have now shown that even a simple strategy, such as size-separation using conventional agarose gel electrophoresis and subsequent PCR analysis (21), can lead to the selective enrichment of circulatory fetal DNA sequences. These, in turn can be used for the determination of DNA polymorphisms that are masked by maternal sequences in the native plasma sample.

MATERIALS AND METHODS

Southern Blot Analysis

Plasma sample collection: Following approval by the Cantonal Institutional Review Board of Basel, Switzerland, 18ml peripheral blood was obtained after written informed consent from pregnant women. EDTA (Sarstedt Movovette tubes; Sarstedt, Sevelen, Switzerland) was used as anti-coagulant. The blood samples were first cleared by centrifugation at 1600g for 10 minutes, following which the plasma was further subjected to a second centrifugation step of 16000g for 10 minutes. Plasma was immediately used for DNA extraction in each analysis.

Southern hybridisation: Circulatory DNA was extracted from approximately 7-10ml maternal plasma (gestational age 11-17 weeks) and 18ml cord blood plasma using a conventional phenol-chloroform procedure, with a slight modification in that the plasma sample was first treated with a chaotropic Guanidium Isothiocyanate solution to denature any nucleases (Qiagen, Basel, Switzerland) (22). As a control, we used plasma from non-pregnant women.

The extracted DNA was separated on a 1.0% agarose gel. A 100bp ladder and Hind III digested Lambda phage DNA were used to estimate molecular size (New England Biolabs, USA). DNA was transferred onto nylon membranes (Roche, Basel, Switzerland) with 20xSSC using standard capillary transfer method (22).

Detection of transferred DNA was performed using the Roche ® DIG labelling and detection system according to the manufacturer's instructions (Roche, Switzerland). The highly repetitive Alu sequence was used as a hybridisation probe, and was directly DIG-labelled by PCR process using PCR DIG Probe Synthesis Kit (Roche, Switzerland). The primer of the Alu sequence is as follows: ATC TCG GCT CAC TGC AA. Prehybridization was carried out at 42°C in DIG Easy Hyb solution (Roche, Switzerland). Hybridization was performed at 42°C overnight and washed at high stringency and incubated with the chemiluminescent alkaline phosphatase substrate (CSPD) according to the manufacturer's instructions and the resulting blot was exposed on X-ray film.

Determination of the size distribution of circulatory fetal-derived DNA in maternal plasma

Circulatory DNA preparation: Plasma samples were prepared as described above. Peripheral blood was collected from the pregnant women carrying a singleton male fetus. Six samples were obtained early in pregnancy (median gestational age = 13+2 weeks) and 8 samples were collected in third trimester (median gestational age = 34+4 weeks). Blood from three non-pregnant women and three healthy males was used as controls. Routinely 5-7 ml plasma was used for DNA extraction, using a combination of the Roche ® High Pure Template DNA Purification Kit (Roche, Switzerland) and a custom made vacuum pump for the isolation of the circulatory plasma DNA. In brief, as described by the manufacturer, the plasma sample was incubated with binding buffer and proteinase K at 70°C for 10 minutes after which the required volume of isopropanol was added and the sample was passed through the Roche ® column under the application of vacuum. Following this, the column was washed with inhibitor removal buffer and twice with wash buffer, respectively, as recommended by the manufacturer. The column bound circulatory DNA was eluted in 40µl elution buffer.

Gel electrophoresis and isolation of circulatory DNA fragments: The total circulatory DNA was subjected to agarose gel electrophoresis using a 1.0% agarose gel (Invitrogen, Basel, Switzerland) containing 0.5µg/ml ethidium bromide (Sigma, USA). Size markers were 100bp ladder and HindIII digested Lambda phage DNA (New England Biolabs, USA). Electrophoresis was carried out at 80V for 1h. Each lane of the gel containing circulatory DNA was then cut using a sterile scalpel blade into six discrete fragments using the molecular weight markers as a guide. The size of the fragments were 0.09-0.3kb, 0.3-0.5kb, 0.5-1.0kb, 1.0-1.5kb, 1.5-23kb, and greater than 23kb. As the 23 kb marker is imprecise in 1% gels, it can only be used as a rough guide to estimate the size of DNA fragments which have sizes greater than 10 kb. The circulatory DNA was extracted from the agarose sections using a QIAEX®II

Gel Extraction Kit (Qiagen, Basel, Switzerland) and eluted in 40µl sterile 10mM Tris-HCl (pH 8.0). Numerous anti-contamination procedures were used during these experiments including: UV irradiation of the gel tray and tank, fresh buffers with each electrophoretic run, as well as the examination of plasma samples from women carrying female fetuses (n=2), and blank gel slices (n= 14) examined parallel with each analysis. In no instance were any false positive results recorded.

Determination of the proportion of circulatory fetal and total DNA: The relative proportions of fetal and total circulatory DNA eluted from the individual agarose gel sections were determined using a well established Taqman ® real time PCR assay for the SRY gene on the Y chromosome and the ubiquitous GAPDH gene (6). The only modifications being that a new generation Perkin Elmer Applied Biosystems 7000 Sequence Detector was used and that MGB (minor groove binding) probes were used, instead of the previous TAMRA conjugated probes (Applied Biosystems, Rotkreuz, Switzerland). The PCR reactions were carried out in a final reaction volume of 25 µL, which consisted of 6µL eluted DNA, 300nmol/L of each primer, 150nmol/L of each probe and 2xTaqMan Universal PCR Master Mix (Applied Biosystems, Rotkreuz, Switzerland). PCR was carried out using an initial incubation at 50°C for 2 minutes to activate Uracil-N-glycosylase, then incubation at 95°C for 10 minutes and followed by 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. This analysis permitted the determination of the total amount of DNA present in each fraction by the use of the GAPDH specific assay, and the fraction of that which was fetal using the SRY specific assay. The relative proportions of each have been expressed as percentages.

Detection of highly polymorphic microsatellite markers

Sample preparation: 18ml maternal blood was collected from three third trimester pregnancies, and cord blood was collected subsequently after delivery. Following plasma separation by high-speed centrifugation, the buffy coat was

collected, washed with PBS (phosphate buffered saline) and used for the preparation of maternal genomic DNA. Fetal genomic DNA was prepared similarly from the cord blood sample. In order to verify that this same approach can also be used to examine clinically relevant samples, we examined 4 samples taken early in pregnancy (median = 13+5 weeks). In this instance the fetal genotype was determined from archived amniocyte or chorionic villus cultures. One of these samples was obtained from a trisomy 21 fetus.

Fluorescent PCR analysis of highly polymorphic microsatellite markers:

The same standardized fluorescent PCR assay for highly polymorphic short tandem repeat (STR) markers on chromosome 21 as used previously for our analysis of urinary DNA was used (24). The fetal and maternal genomic DNA sources were used to determine which short tandem repeat (STR) makers on chromosome 21 were informative. These informative STR markers were then used for the detection of paternally and maternally inherited fetal alleles in total maternal plasma circulatory DNA, as well as in size-separated circulatory DNA fractions. The preparation of the low molecular weight circulatory DNA fraction was carried out as described above.

As the concentration of circulatory DNA following size fractionation was very low, use had to be made of a semi-nested PCR assay, as described previously (24). The PCR products were analysed by capillary electrophoresis on a ABI 310 gene analyser (Applied Biosystems). For the analysis of the samples taken early in pregnancy, where the concentration of circulatory fetal DNA has been shown to be lower than at term, our investigation showed that the PCR procedure had to be modified in order to obtain optimal results. For this reason, we used a “touch-down” PCR method instead of our conventional semi-nested PCR method described above. In this procedure, a total of 50 cycles were run, using PCR reactions containing 3ul of 10X buffer; 3.5mM MgCl₂; 160μM dNTPs; 0.1μM each of forward and reverse primers (24) (one primer of each set was fluorescent labelled); 1U of AmpliTaq Gold polymerase and 3μL of DNA in final 30μL volume. After incubation at 95°C for 10min, 10 cycles (95°C for 15s, 65°C for 15s, 72°C for 30s) of thermal cycling were carried out whereby the annealing temperature was decreased 1°C every cycle. This was then followed by 40 further cycles of 95°C for 15s, 56°C for 15s,

and 72°C for 30s. The PCR terminated following an extension phase at 72°C for 7min. As described above, the PCR products were analysed by capillary electrophoresis on a ABI 310 gene analyser (24).

RESULTS

Circulatory DNA has been proposed to exhibit apoptotic hallmarks such as oligonucleosomal laddering and nucleosome association (25, 26). In our initial investigation we attempted to determine whether circulatory DNA in pregnant women also displayed such characteristics. For this examination we used Southern blot analysis of total circulatory plasma DNA that had been subjected to agarose gel electrophoresis. The analysis of such blots using the ubiquitous highly repetitive Alu sequence, indicated that oligonucleosomal fragments could indeed be detected, and were present in all three of the plasma sources examined: maternal blood, non-pregnant female control and cord blood (refer to figure 1). This examination also indicated that a significant proportion of the circulatory DNA had a molecular size of larger than 10 or even 23 kb (refer to figure 1). The presence of such high molecular weight DNA species cannot be attributed to the plasma sample being contaminated by maternal cells, as extreme care was taken to obtain cell free plasma samples. It is of interest that these high molecular weight DNA molecules are quite similar to those the very large ones we observe in terminally differentiated erythroblasts prior to enucleation (Hristoskova et al., manuscript in preparation).

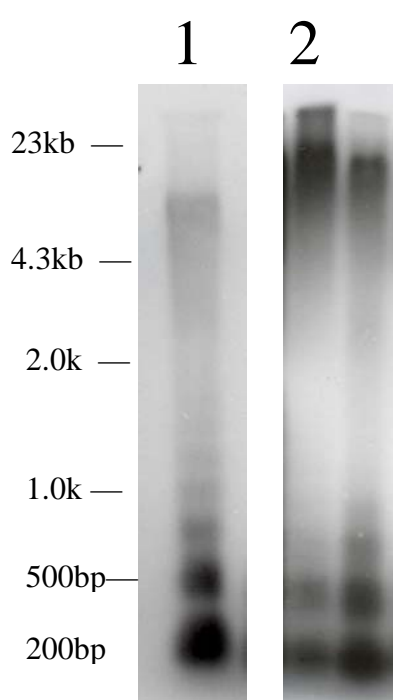


Fig. 1. Southern blot analysis of plasma circulatory DNA, using a highly repetitive Alu probe.

Lane 1, plasma from cord blood;
lane 2, maternal plasma (13 weeks of gestation);
lane 3, plasma from nonpregnant woman.

Unfortunately we were not able to determine the characteristics of circulatory fetal DNA in the samples obtained from pregnant women, as the concentrations of fetal DNA proved to be too low to be detectable, even when using a very high copy (DYS14) probe specific for the Y chromosome.

As we were, however, able to detect circulatory apoptotic DNA fragments in our Southern blot analysis, we used an alternative strategy to determine whether circulatory fetal DNA displayed a similar pattern. For this analysis, we used an approach that had previously been successfully used for the characterisation of rare linear extra-chromosomal DNA species (21). In this procedure, the circulatory DNA was first subjected to agarose gel electrophoresis following which individual gel fragments containing the size-fractionated DNA were examined by PCR. For our examination, following electrophoresis, the agarose gel was cut into six discrete fragments having approximate sizes of <0.3kb, 0.3 – 0.5kb, 0.5-1.0 kb, 1.0 – 1.5kb, 1.5 – 23.0 kb and > 23 kb. Once the circulatory DNA was extracted from these gel fragments, the proportion of fetal and maternal DNA in these fractions was then determined using well established real-time PCR assays for the SRY locus on the Y chromosome and the ubiquitous GAPDH (glyceraldehyde 3-phosphate dehydrogenase) gene (6). To ensure that we were not being misled by any PCR artefacts, we included numerous anti-contamination procedures in our study, including plasma samples from women pregnant with female fetuses (n=2) and the parallel examination of blank gel slices in each analysis (n=14). No false positive results were recorded in any of these instances.

Our examination of plasma samples from third trimester pregnancies with a male fetus indicated that the vast proportion of circulatory fetal DNA, as detected by the SRY specific PCR assay, had an approximate molecular size of less than 300bp (refer to table 1 and figure 2), with very little or no fetal DNA having a molecular size of greater than 1 kb. Maternally derived sequences, as determined by the GAPDH specific PCR assay, on the other hand were estimated to be larger than 0.5 to 1.0 kb and included molecular weight species larger than 10 to 20 kb. A similar pattern for both fetal and maternal circulatory DNA was also observed in plasma samples obtained early in the second trimester of pregnancy (table 2, figure 3).

Table 1. Size distribution of total and fetal circulatory DNA in third-trimester maternal plasma samples.^a

Size of DNA fraction, kb	Median (range)		
	Size distribution of total DNA, ^b %	Size distribution of fetal DNA, ^c %	Proportion of fetal DNA per fraction, ^d %
<0.3	22.4 (15.7–26.7)	70.0 (51.0–82.3)	68.7 (22.2–87.1)
0.3–0.5	28.4 (15.7–35.2)	24.3 (13.8–31.6)	15.4 (6.4–31.4)
0.5–1.0	23.0 (15.0–26.8)	3.8 (0.0–17.4)	2.6 (0.0–7.8)
1.0–1.5	7.5 (2.2–11.4)	0.0 (0.0–8.7)	0.0
1.5–23	21.1 (10.3–35.7)	0.0	0.0

^a Six samples were analyzed in this study. Median gestational age was 34 (4) weeks.

^b Size distribution of total circulatory DNA was determined by a real-time PCR assay for the *GAPDH* gene. The values are indicative of the percentage of total DNA in each fraction examined.

^c Size distribution of circulatory fetal DNA as determined by a real-time PCR assay for the *SRY* gene. These values are indicative of the percentage of fetal DNA with regard to the total amount of fetal DNA in each fraction examined.

^d The proportion of fetal DNA indicates the percentage of fetal DNA in each of the examined fractions with regard to the total amount of circulatory DNA in that fraction.

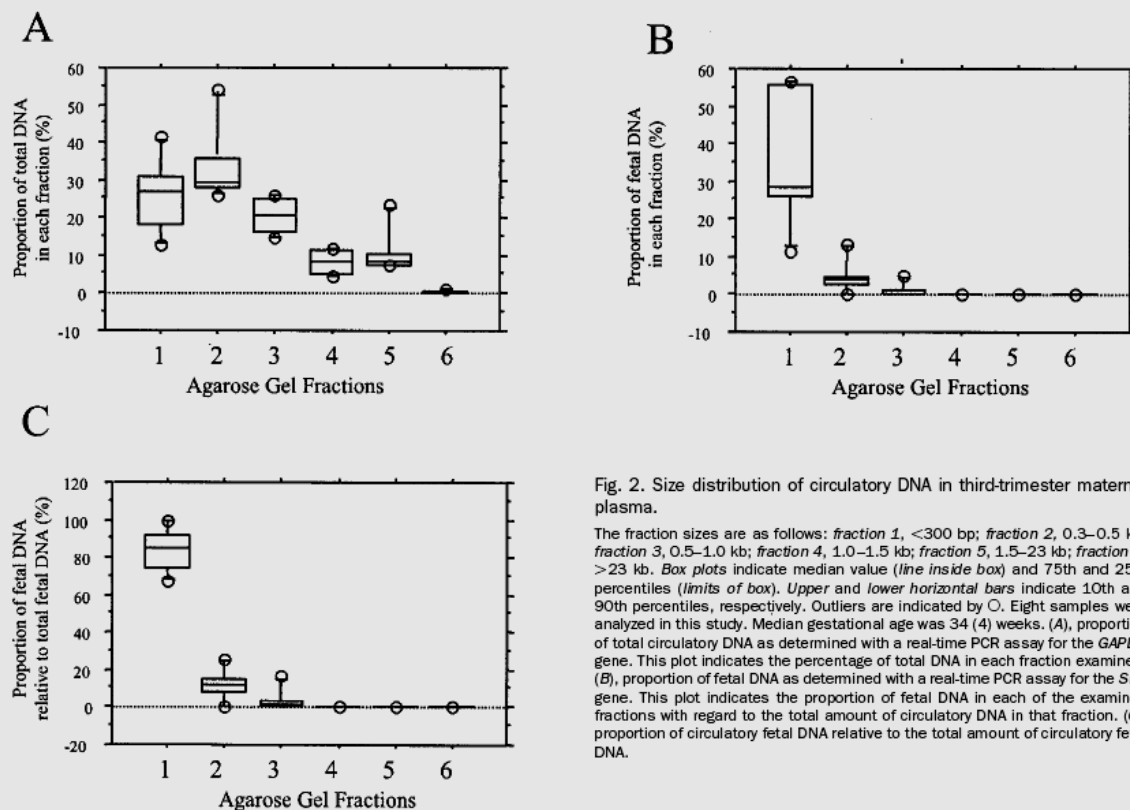


Fig. 2. Size distribution of circulatory DNA in third-trimester maternal plasma.

The fraction sizes are as follows: fraction 1, <300 bp; fraction 2, 0.3–0.5 kb; fraction 3, 0.5–1.0 kb; fraction 4, 1.0–1.5 kb; fraction 5, 1.5–23 kb; fraction 6, >23 kb. Box plots indicate median value (line inside box) and 75th and 25th percentiles (limits of box). Upper and lower horizontal bars indicate 10th and 90th percentiles, respectively. Outliers are indicated by O. Eight samples were analyzed in this study. Median gestational age was 34 (4) weeks. (A), proportion of total circulatory DNA as determined with a real-time PCR assay for the *GAPDH* gene. This plot indicates the percentage of total DNA in each fraction examined. (B), proportion of fetal DNA as determined with a real-time PCR assay for the *SRY* gene. This plot indicates the proportion of fetal DNA in each of the examined fractions with regard to the total amount of circulatory DNA in that fraction. (C), proportion of circulatory fetal DNA relative to the total amount of circulatory fetal DNA.

Table 2. Size distribution of total and fetal circulatory DNA in maternal plasma samples obtained early in pregnancy.^a

	Median (range)		
Size of DNA fraction, kb	Size distribution of total DNA, %	Size distribution of fetal DNA, %	Proportion of fetal DNA per fraction, %
<0.3	26.9 (12.7–41.3)	85.5 (67.8–100.0)	28.4 (11.6–56.6)
0.3–0.5	29.1 (26.1–54.4)	11.7 (0.0–15.5)	4.0 (0.0–13.5)
0.5–1.0	28.5 (14.5–25.9)	1.2 (0.0–16.8)	0.4 (0.0–5.2)
1.0–1.5	8.2 (4.5–12.1)	0.0	0.0
1.5–23	8.6 (7.6–23.7)	0.0	0.0

^a Eight samples were analyzed in this study. Median gestational age was 13 (2) weeks. Size distributions of total circulatory DNA, circulatory fetal DNA, and the proportion of fetal DNA were determined as described in the footnotes for Table 1.

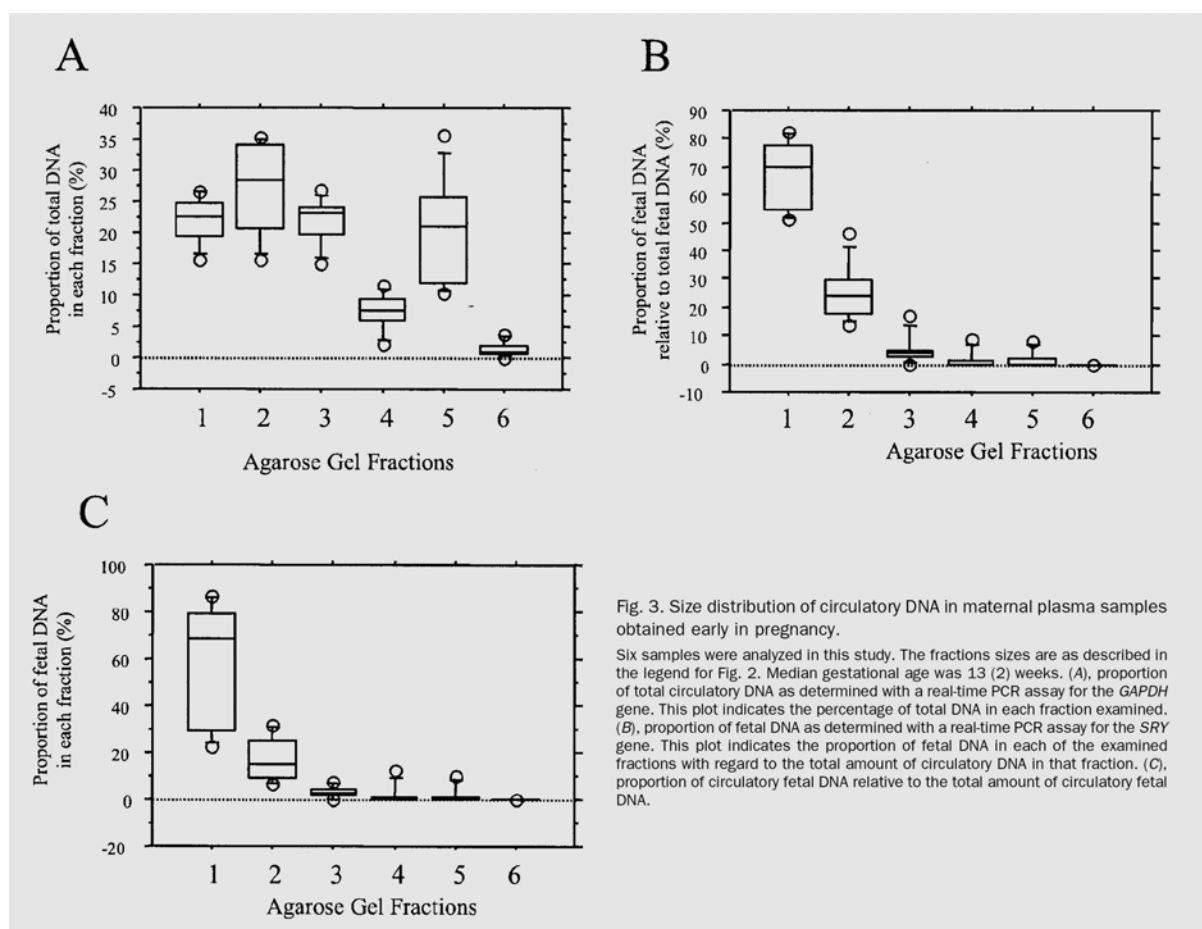
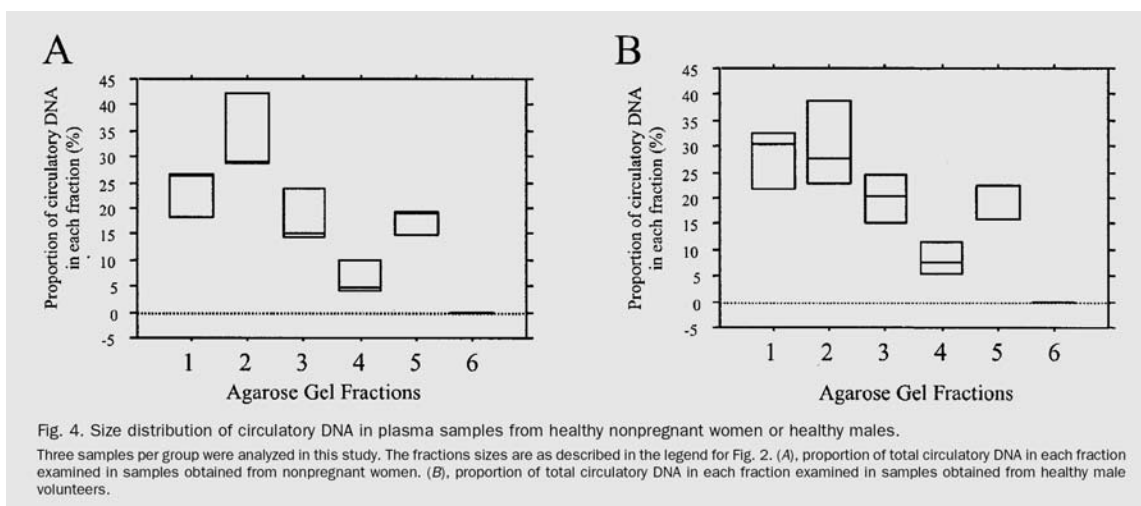


Fig. 3. Size distribution of circulatory DNA in maternal plasma samples obtained early in pregnancy.

Six samples were analyzed in this study. The fractions sizes are as described in the legend for Fig. 2. Median gestational age was 13 (2) weeks. (A), proportion of total circulatory DNA as determined with a real-time PCR assay for the *GAPDH* gene. This plot indicates the percentage of total DNA in each fraction examined. (B), proportion of fetal DNA as determined with a real-time PCR assay for the *SRY* gene. This plot indicates the proportion of fetal DNA in each of the examined fractions with regard to the total amount of circulatory DNA in that fraction. (C), proportion of circulatory fetal DNA relative to the total amount of circulatory fetal DNA.

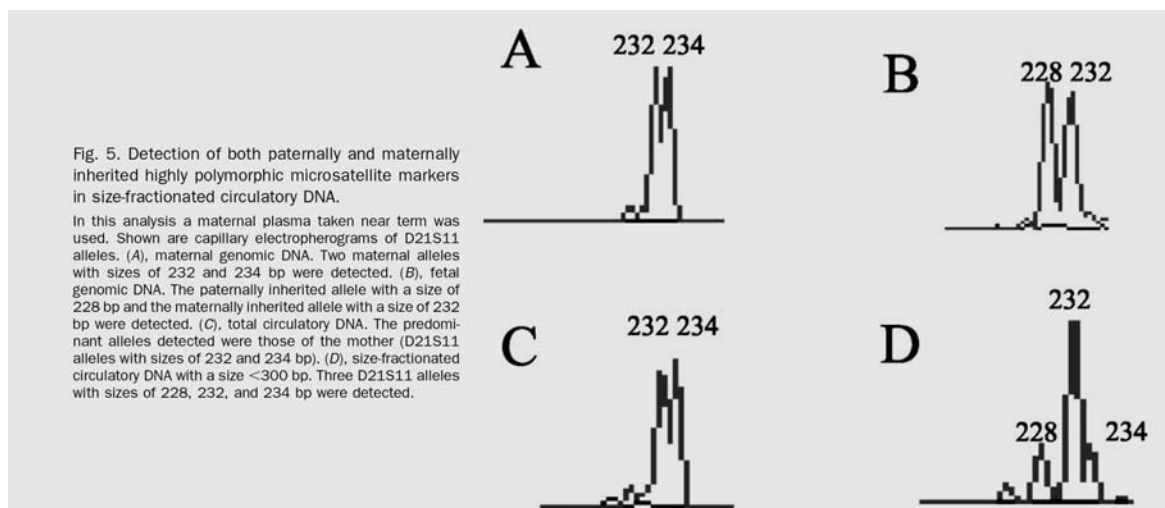
With regard to the size distribution of total circulatory DNA, we determined that the pattern we had observed in pregnant women was very similar to that observed in samples taken from non-pregnant women as well as healthy male volunteers (figure 4). In none of these analyses were we able to detect large amounts of DNA with a molecular size greater than that indicated by the 23kb molecular weight marker, in contrast to what we observed in our Southern blot analysis (figure 1). The reason for this anomaly may be that these large fragments are not easily eluted from the agarose gel under the conditions we are using, unlike in the Southern blotting where the DNA is first treated with alkali in order to generate the small fragments required for efficient capillary transfer.



Our data also indicated that by examining DNA fragments of a size less than approximately 300bp, a selective enrichment of circulatory fetal DNA sequences may be possible to achieve. Our next step was, therefore, to determine whether such size dependent separation would facilitate the determination of more subtle fetal genetic traits.

For this purpose, we examined whether both paternally and maternally inherited DNA polymorphisms could be discerned from such size fractionated circulatory DNA. For our analysis we used highly polymorphic short tandem repeat (STRs) sequences on chromosome 21, which have previously been demonstrated to be suitable for the reliable distinction of mother and child (24). In order to test the feasibility of this approach we first examined

samples taken close to term, as here the concentration of circulatory fetal DNA is at a maximum, and since it was readily possible to obtain the fetal genotype, a prerequisite for such studies, from a cord blood sample after birth. In this manner, we could select easily discernible polymorphic markers between mother (Fig 5a) and fetus (Fig. 5b).



In this analysis, the benefits of selectively enriching for circulatory fetal DNA species becomes readily apparent, in that the paternally inherited STR allele (with a size of 228bp) is barely detectable in total plasma extracted DNA (figure 5C), but is clearly present in the DNA fraction with a size of less than 300bp (figure 5D). Furthermore, the maternally inherited STR allele (with a size of 232bp) cannot be distinguished from the predominantly maternal pattern obtained from the analysis of total plasma extracted DNA (figure 5C). This fetal allele can, however, be detected in the analysis of the DNA fraction with a size of less than 300bp (figure 5D), as here the peak for that STR allele has a much greater area than either the paternally inherited fetal allele (228bp) or the solitary maternal allele (234bp). This indicates that both fetal and maternal loci are contributing to the presence of this particular PCR product. Similar results were obtained in the analysis of the DNA fraction with a size between 300 to 500bp, although the results in this instance were less refined (data not shown). The reproducibility of this approach was verified in the analysis of a further 2 samples, which were analysed at a number of different polymorphic loci, where analogous results were obtained (refer to table 3).

Table 3. Detection of paternally and maternally inherited fetal highly polymorphic microsatellite markers in size-fractionated circulatory DNA obtained from maternal plasma samples taken close to term.^a

Case no. (D21 locus)	Method of sample preparation ^b	Maternal alleles detected	Fetal alleles detected
1 (D21S11)	Maternal genomic DNA	232/234	
	Fetal genomic DNA		228/232
	Total plasma DNA	232/234	Not detectable
	Plasma DNA <300 bp	232/234	228/232
1 (D21S1435)	Maternal genomic DNA	172/180	
	Fetal genomic DNA		172/176
	Total plasma DNA	172/180	176 ^c
	Plasma DNA <300 bp	172/180	172/176
2 (D21S1270)	Maternal genomic DNA	184/188	
	Fetal genomic DNA		180/184
	Total plasma DNA	184/188	Not detectable
	Plasma DNA <300 bp	184/188	180/184
2 (D21S1432)	Maternal genomic DNA	138/152	
	Fetal genomic DNA		134/138
	Total plasma DNA	138/152	134 ^c
	Plasma DNA <300 bp	138/152	134/138
3 (D21S1435)	Maternal genomic DNA	168/176	
	Fetal genomic DNA		168/172
	Total plasma DNA	168/176	172 ^c
	Plasma DNA <300 bp	168/176	168/172

^a Three samples were analyzed in this study.

^b Genomic DNA was prepared directly from maternal or fetal lymphocytes. Total plasma DNA indicates analysis of circulatory DNA extracted from an nonfractionated DNA samples, whereas plasma DNA <300 bp indicates analysis of a discrete fraction of circulatory DNA that had been size-fractionated by gel electrophoresis.

^c Minimal detection of paternally inherited allele.

In order to determine whether this approach could also be applied to clinically relevant samples, we also examined four samples taken early in the second trimester of pregnancy. In these analyses the fetal genotype was determined from archived amniocyte or chorionic villus cultures. One of these samples was from a fetus affected by Down's syndrome (trisomy 21). This is evident from our microsatellite analysis for the D21S1432 marker, where three equivalent

peaks with sizes of 133, 137 and 141bp respectively are recorded (Fig 6b), implying that the fetus has inherited a copy of each of the maternal chromosome 21's, in addition to the paternally inherited chromosome 21. In our analysis of the total circulatory cell free DNA in the maternal plasma sample, only the two maternal markers can be readily discerned (133 and 141bp) (Fig 6c), whereas our analysis of size – fractionated DNA having an approximate size of less than 300bp facilitates the ready detection of the paternally inherited 137bp marker (Fig 6d). A similar feature was observed when examining this sample for a different microsatellite marker, namely D21S1270 (Table 4). In this instance, it was not possible to determine whether the maternally inherited polymorphisms could be detected, as the fetus has the same pattern as the mother for both loci. In the three other cases, which had a normal karyotype, we were in all three cases readily able to detect the paternally inherited polymorphic locus in the size fractionated DNA sample (Table 4). However, only in one instance could we discern the presence of the maternally inherited locus (Case 3; Table 4). In case 2, this was not possible as the mother was homozygous for the locus interrogated, whereas in the other cases (case 2: D21S1435 and case 4: D21S1440), the maternal locus which had not been inherited by the fetus appeared to have been preferentially amplified (Table 4).

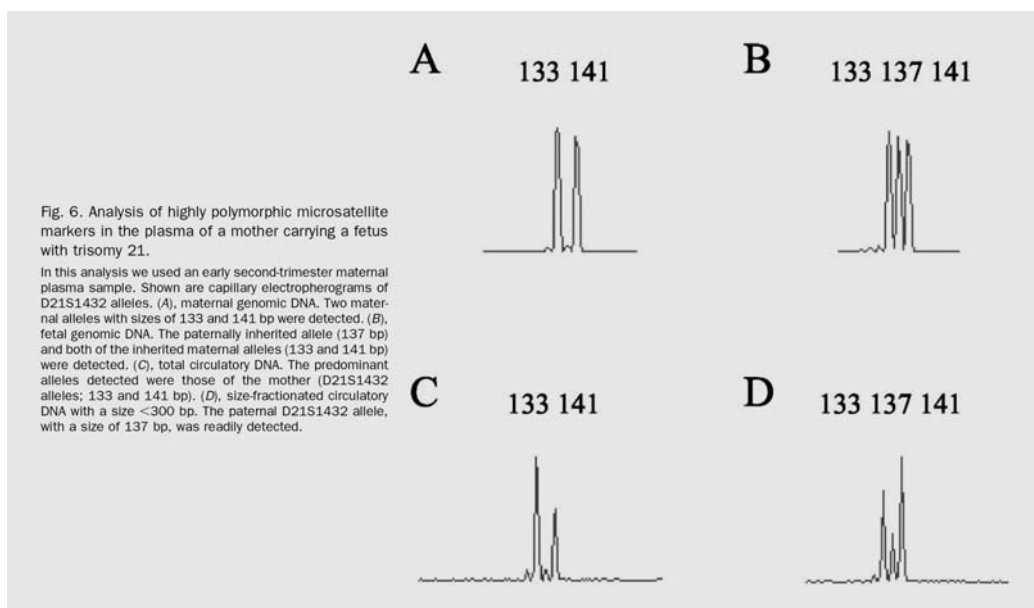


Table 4. Detection of paternally and maternally inherited fetal highly polymorphic microsatellite markers in size-fractionated circulatory DNA obtained from maternal plasma samples obtained early in the second trimester.^a

Case no. (D21 locus)	Method of sample preparation ^b	Maternal alleles detected	Fetal alleles detected
1 (D21S1270)	Maternal genomic DNA	182/191	
	Fetal genomic DNA		182/187/191
	Total plasma DNA	182/191	Not detectable
	Plasma DNA <300 bp	182/191	187
1 (D21S1432)	Maternal genomic DNA	133/141	
	Fetal genomic DNA		133/137/141
	Total plasma DNA	133/141	Not detectable
	Plasma DNA <300 bp	133/141	137
2 (D21S1440)	Maternal genomic DNA	154	
	Fetal genomic DNA		154/157
	Total plasma DNA	154	Not detectable
	Plasma DNA <300 bp	154	157
2 (D21S1435)	Maternal genomic DNA	141/172	
	Fetal genomic DNA		172/176
	Total plasma DNA	141/172	Not detectable
	Plasma DNA <300 bp	141/172	176
3 (D21S1440)	Maternal genomic DNA	157/160	
	Fetal genomic DNA		154/157
	Total plasma DNA	157/160	Not detectable
	Plasma DNA <300 bp	157/160	154/157
4 (D21S1440)	Maternal genomic DNA	154/157	
	Fetal genomic DNA		157/160
	Total plasma DNA	154/157	Not detectable
	Plasma DNA <300 bp	154/157	160

^a Four samples were analyzed in this study.

^b Genomic DNA was prepared directly from maternal or fetal lymphocytes. Total plasma DNA indicates analysis of circulatory DNA extracted from an nonfractionated DNA samples, whereas plasma DNA <300 bp indicates analysis of a discrete fraction of circulatory DNA that had been size-fractionated by gel electrophoresis.

DISCUSSION

Our investigation supports the current hypothesis that circulatory DNA has apoptotic attributes (25, 26), in that we can readily discern oligonucleosomally cleaved fragments using Southern blot analysis. Our analysis also indicates that a significant proportion of the circulatory DNA has a very large molecular size of greater than 20 kb. Independent investigations in our laboratory indicate that these large circulatory DNA species may be derived from the erythropoietic system, in that DNA isolated from terminally differentiating erythroblasts exhibits similar characteristics (Hristoskova et al., manuscript in preparation). It is currently unclear whether these large DNA molecules are subsequently cleaved into smaller oligonucleosomal fragments in the maternal plasma, or whether the smaller fragments we have detected are derived from another source.

By using an approach which had previously been used to examine rare linear extra-chromosomal DNA species (21), whereby DNA size fractionated by gel electrophoresis is subsequently extracted and analysed by PCR, we made the surprising finding that a large discrepancy existed in the size of circulatory fetal and maternal DNA species. In this regard our study indicated that fetal DNA molecules predominantly have an approximate size of 300bp or less, whereas most maternally derived DNA molecules are considerably larger than this. The fact that no large circulatory fetal DNA species are detected (i.e. greater than 20 kb) implies that the mechanism contributing to the formation of the large maternally derived DNA species is not involved in the liberation of circulatory fetal DNA. The explanation for this difference could be that circulatory fetal DNA appears to be exclusively derived from the placenta (27), whereas the vast proportion of normal circulatory DNA is of hemopoietic origin (28).

With regard to the size distribution of circulatory maternal and fetal DNA species, our results are remarkably similar to a recent observation made by Chan and colleagues (20), who also observed that cell fetal DNA molecules were generally smaller than those of maternal origin. For their study, they made use of differently sized PCR amplicons ranging in size from 105bp to

798bp for the size determination of predominantly maternal derived DNA molecules, and 107 to 524bp for the examination of circulatory fetal DNA molecules. Although their approach permitted a much more precise delineation of the size distribution of circulatory DNA molecules within this given range, this study was not able to demonstrate the existence of very large (>10 to 20 kb) maternal circulatory DNA species in the way that our analysis facilitated.

A further important point that the study of Chan (20) did not address, and which has been the major focus of our study, is that this observation permits the development of a strategy facilitating the selective enrichment of circulatory fetal DNA sequences. The exploitation of this approach, in turn permits the determination of highly polymorphic fetal genetic traits not discernible from the analysis of total plasma extracted circulatory DNA. In this manner we were able to detect the presence of both paternally and maternally inherited STR markers in size separated circulatory DNA fractions, a feature which was not possible when attempting the same analysis on un-fractionated samples (13, 14). Our study does, however, indicate that the method we have chosen for this proof-of-concept study is too imprecise and inefficient to be used for potential clinical applications. This becomes clear in our analysis of paternally and maternally inherited polymorphic markers, where it is not possible to determine the precise proportion that the fetal markers contribute to the analysed pattern. This feature is very important, as numerous clinical studies have clearly shown that the analysis of such highly polymorphic STR markers is very useful for the analysis of fetal ploidy (15). Hence, if our approach using size-fractionation were functioning in an optimal manner, it should be feasible to determine fetal chromosomal anomalies directly from maternal plasma. As our study included one case with a trisomy 21 fetus, where we were not able to determine fetal ploidy from the size fractionated DNA sample (Figure 6; Table 4), this application will have to await developments, which permit a more efficacious separation of maternal and fetal DNA species. It is, however, possible, that even in its current form, that our approach could be used for the non-invasive determination of paternity.

A further important aspect of our observation is that it may in future aid in the examination of Mendelian disorders, particularly those involving point mutations, as these analyses should no longer be hindered by the vast excess

of circulatory maternal DNA sequences (2, 3). Indeed, by the use of quantitative assays it may even be possible to determine the fetal genotype in those instances where both partners are carriers for the same disease allele.

In summary, our finding has shown that circulatory fetal DNA molecules are generally of a smaller size than comparable maternally derived sequences, and that a selective enrichment of fetal DNA sequences can be achieved using size dependent separation. This latter feature permits the detection of fetal genetic traits not possible from total plasma circulatory DNA. For clinical applications, more efficacious separation modes will need to be developed which facilitate a better discrimination and recovery of fetal and maternal circulatory DNA species.

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Detection of Paternally Inherited - Globin Gene Mutations by the Use of Size-Fractionated Cell-Free DNA in Maternal Plasma.

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Chapter 9

Summary

Pregnancies at risk for compound heterozygous single gene disorders, such as the hemoglobinopathies, could be screened for by detection of paternally inherited mutations. Although fetal DNA can be readily detected in maternal plasma, this approach cannot currently be used for the reliable analysis of fetal mutant alleles involving single nucleotide changes, as these are masked by circulatory maternal DNA. It has recently been described that size-separation of plasma DNA permits the ready detection of fetal genetic traits. We now show this method can be used for the reliable determination of paternal inheritance of three common point mutations for β thalassemia, namely IVSI-110, IVSI-1 and IVSI-6.

β thalassemia, characterized by mutations of the β globin gene, is a common monogenic disorder ¹. Recently fetal DNA in maternal plasma has been used for the assessment of pregnancies at risk for β thalassemia major by the determination of fetal inheritance of the paternal mutation codon 41/42 ². This analysis can be reliably performed as this large 4 base deletion is easily distinguished from the normal maternal allele. The similar analysis of fetal mutations involving single base changes, which constitute the majority of genetic lesions in the hemoglobinopathies, is more complex due to the preponderance of maternal DNA sequences.

It has recently been shown that circulatory fetal DNA is generally smaller than maternal DNA ^{3,4}, and that size-fractionation can be used for a selective enrichment of fetal DNA sequences which permits the detection of otherwise masked fetal DNA sequences ⁴.

We have now examined whether this approach can be used for the determination of paternally inherited β globin mutations from maternal plasma. In our study, we examined three mutations of the β globin gene which involve single nucleotide exchanges, IVSI-110 (G-A), IVSI-1 (G-A) and IVSI-6 (A-G), which significantly contribute to β thalassemia cases in the Mediterranean area ¹. Following institutional ethical approval and informed consent, blood samples (n=16) were obtained in Bari, Italy from pregnancies at risk for β thalassemia, where the father was a carrier for one of the three mutations described above and the mother carried another β globin mutation. Samples were collected at approximately 12 weeks of gestation prior to an invasive procedure, and either whole blood (n=8; shipped at room temperature) or the plasma (n=8; at -20°C) was sent to Basel for blinded analysis.

Following extraction and size-fractionation ⁴, the DNA was first amplified in a PCR reaction with the PNA (peptide nucleic acid) clamp, which ensures optimal detection of the paternal mutant allele by suppressing erroneous amplification of the normal allele ⁵. Detection of the paternal mutant allele, was then accomplished by allele-specific real-time PCR (Table 1).

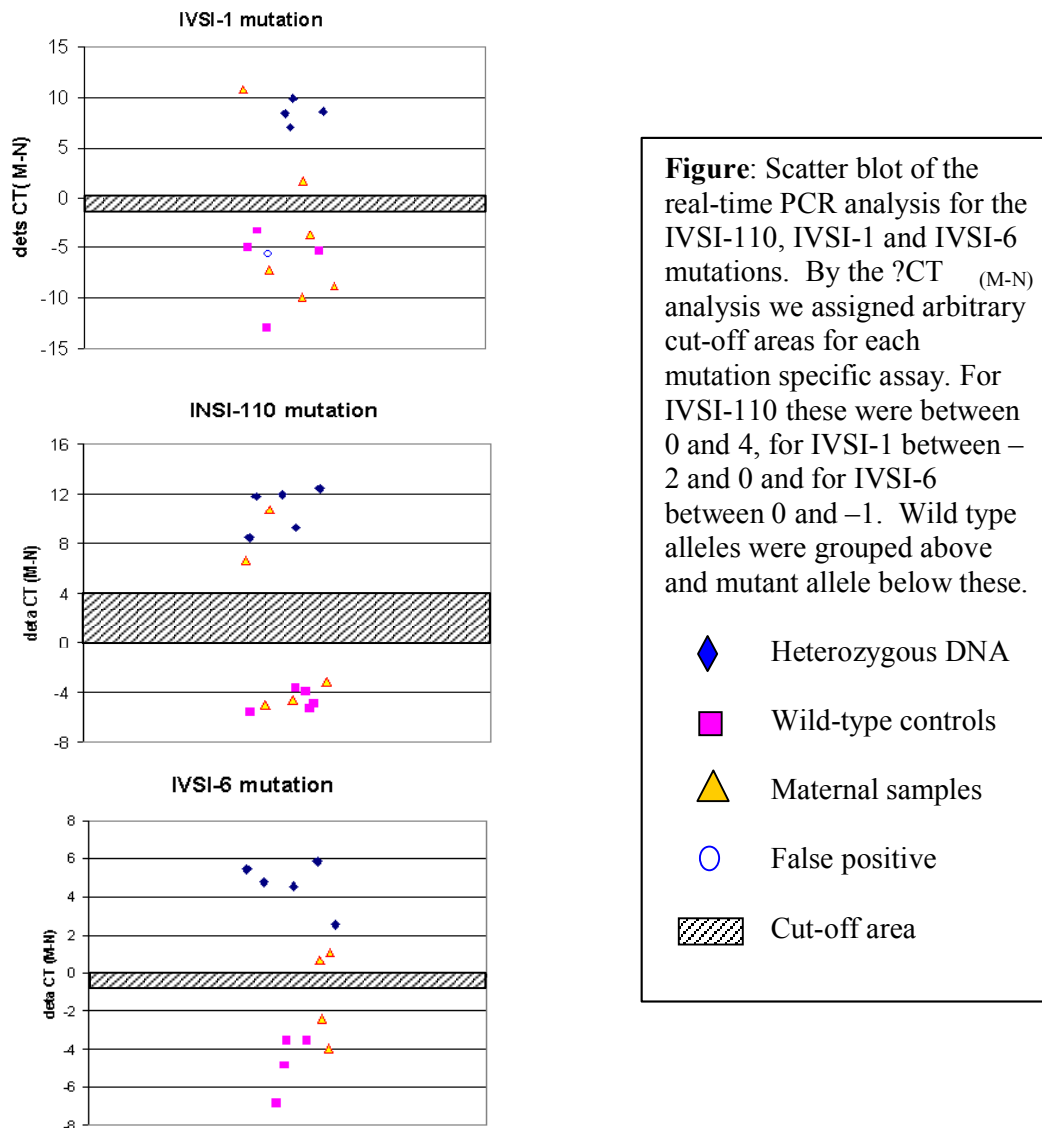
Mutations	Primers and PNA probes	The conditions of PNA-Clamping and Real time-allele specific -PCR
IVSI-110	PNA-Clamping: F-Primer: ACT CTT GGG TTT CTG ATA GGC ACT R-Primer: CAG CCT AAG GGT GGG AAA ATA G PNA probe: OO-TAG ACC AAT AGG C Allelic specific-real time PCR: F-Primer: ACT CTT GGG TTT CTG ATA GGC ACT IVSI-110/N: CAG CCT AAG GGT GGG AAA ATA CAC C IVSI-110/M: CAG CCT AAG GGT GGG AAA ATA CAC T	Denatured at 95°C 10min, followed by 25cycles of 95°C 15sec, 71°C for 1min, 62°C for 15sec, 72°C for 30sec, final extension at 72°C for 5min Incubated at 95°C for 10min, followed by 40cycles of 95°C for 15sec, 59.5°C for 15sec, 72°C for 30sec
IVSI-1	PNA-Clamping: F-Primer: GTG AAC GTG GAT GAA GTT GGT R-Primer: TCT CCT TAA ACC TGT CTT GTA ACC TTC TAT PNA probe: OO-GAT ACC AAC CTG CCC Allelic specific-real time PCR: F-Primer: GTG AAC GTG GAT GAA GTT GGT IVSI-1/N: TTA AAC CTG TCT TGT AAC CTT GAT ACG AAC IVSI-1/M : TTA AAC CTG TCT TGT AAC CTT GAT ACG AAT	Denatured at 95°C for 10min, followed by 25cycles of 95°C for 15sec, 70°C for 1min, 60°C for 15sec, 72°C for 30sec, final extension at 72°C for 5min Incubated at 95°C 10min, followed by 40cycles of 95°C for 15sec, 63.5°C for 15sec, 72°C for 30sec
IVSI-6	PNA-Clamping: F-Primer: GTG AAC GTG GAT GAA GTT GGT R-Primer: CTT AAA CCT GTC TTG TAA CCT TGA PNA probe: OO-GAT ACC AAC CTG CCC Allelic specific-real time PCR: F-Primer: GTG AAC GTG GAT GAA GTT GGT IVSI-6/N: CT TAA ACC TGT CTT GTA ACC TTC TAT IVSI-6/M : CT TAA ACC TGT CTT GTA ACC TTC ATG	Denatured at 95°C for 10min, followed by 25cycles of 95°C for 15sec, 70°C for 1min, 60°C for 15sec, 72°C for 30sec, final extension at 72°C for 5min Incubated at 95°C for 10min, followed by 40cycles of 95°C for 15sec, 62.5°C for 15sec, 72°C for 30sec

Table: Oligonucleotide sequences and PCR conditions.

The PCR clamping reaction was performed in a total volume of 30µL, consisting of 1x buffer with 3.5mM Mg²⁺, 0.2 mM dNTPs, 10µL DNA, 0.13µM of each primer (Microsynth, Switzerland), 0.6 U TaqGoldTM DNA polymerase (Applied Biosystems, Switzerland), using the following PNA probe concentrations (Applied Biosystems): 0.67 µM for the IVSI-1 mutation, 0.5µM of PNA in case of IVSI-6 and 1µM of PNA in case of IVSI-110. The real-time allele-specific PCR was then performed using 1µL of the PCR clamping product in a total volume of 25 µL containing 1x SYBR Green Master Mix and 0.16µM of each primer (Table 1).

For the reliable determination of the paternal mutant allele, we used a ΔCT system, whereby the extent of the amplification of the normal wild-type allele (CT_N) was

subtracted from that of the mutant allele (CT_M). This approach lead to a clear discrimination of normal wild-type DNA samples from those heterozygous for the mutant allele, even under experimental conditions where the mutant allele constituted less than 10% of the total DNA examined. This analysis permitted us to assign arbitrary $\Delta CT_{(M-N)}$ cut-off areas for each of the 3 assays for the segregation of normal samples from those with the mutant allele (Figure 1).



The comparison of our blinded analysis of the maternal plasma samples with result of the invasive procedure indicated that the presence or absence of a paternal mutant allele had been correctly determined in 15 or the 16 cases examined (Figure 1). One false positive result was scored for our analysis of a IVSI-1 mutation (Figure 1). Our

re-analysis of this sample indicated that the total DNA concentration was almost 4 fold lower than the other samples analyzed. Since wild type samples with similar low amounts of total input DNA had the $\Delta CT_{(M-N)}$ values close to 0, it is possible that low template DNA concentrations lead to anomalies during the PCR clamping step. Hence, we recommend that the concentration of input DNA should be within a certain range (50-700 genome-equivalents) for the assay to function reliably.

Although the approach that we have outlined will have to be validated in independent and larger studies, our study underscores the validity of approaches enriching fetal DNA sequences⁴, as our parallel assessment of total plasma DNA samples lead to the incorrect evaluation of 3 of 9 cases which had inherited the paternal mutation.

The advantage of the described method is that it relatively simple and can be performed without the need for complex machinery, as it relies on technologies consistent with those currently used in routine diagnostic laboratories. Furthermore, it can be readily modified to permit a similar non-invasive prenatal assessment of other single gene disorders involving compound heterozygous mutations.

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Optimized Quantitative Measurement of Fetal Male DNA in Plasma by Real-Time PCR.

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Chapter 10

Abstract

Background: The cell free form of fetal DNA can be found in the plasma of pregnant women. The quantitative measurement of fetal DNA by real-time quantitative PCR has been applied in many studies to investigate a correlation between increased levels and pregnancy related disorders. For example, an increase of fetal DNA has been reported in pregnancies with fetal trisomy 21. However, The levels determined are close to the detection limit, and the measurements are performed under PCR conditions that do not allow accurate measurements. Consequently measurements performed by different laboratories lead to results that are not comparable.

Methods: We amended an existing but sub-optimal protocol for the real-time quantitative PCR amplification of the DYS14 sequence, which can be found in multiple copies on the Y-chromosome. By our analysis of 46 plasma samples of pregnancies in the first trimester we show that the method produces better results than currently used single copy sequence targeting protocols.

Results: By our methods, we determined higher circulatory fetal DNA levels than previously reported. In 17 male pregnancies of normal outcome the average fetal DNA content was 426 genome equivalents per ml plasma.

Conclusion: The determination of low copy numbers is affected by reduced and variable amplification. We present a real-time PCR method that increases the robustness of male fetal DNA determination by amplifying a sequence that is present in multiple copies per male genome.

Introduction

Since Lo first described the presence of circulatory free fetal DNA (cffDNA) in the maternal circulation in 1998, it's PCR amplification has emerged as the main strategy for the development of non-invasive methods for prenatal diagnosis. One of the main clinical applications is the prediction of fetal sex by the real-time quantitative PCR technique, as in the assessment of X-linked disorders. Y-chromosome specific sequences are also measured to investigate for a correlation between fetal DNA levels and pregnancy-associated complications, such as trisomies, preterm labor and preeclampsia. The method is only applicable to 50% of the samples due to it's restriction to pregnancies carrying male fetuses.

As the levels of circulatory fetal DNA in the first and second trimester are very low, the quantitation is performed close to the detection limit. The precision and reproducibility of the quantitative measurements can greatly be improved by usage of Y-specific sequences that are present in more than one copy per Y-chromosome. We developed a protocol specific to the multicopy sequence DYS14 and evaluated its performance in comparison to our currently used protocol. Our evaluation shows that the new protocol performs with an increased accuracy and sensitivity. It greatly enhance the quality of studies correlating fetal DNA levels with pregnancy associated pathologies.

Material and Methods

Genomic reference DNA was extracted from male white blood cells, quantified with a spectrophotometer (NanoDrop-1000, Witec AG, Switzerland) and converted into genome equivalents (GE) on the basis of 6.6 pg of DNA per cell (1).

Frozen plasma samples of pregnant women in the 1st trimester were extracted with the Roche (Switzerland) high pure PCR template kit. The manufacturers instructions were amended in that we extracted 400 µl of plasma, used 40 µl proteinase K for digestion, and eluted the DNA into 50 µl of 20% elution buffer (diluted in water). Prior to elution, the columns were pre-warmed in a shaker at 70°C for 5 minutes. Extracted DNA was stored at 4°C.

Real-time quantitative PCR amplification

The real-time qPCR was performed in the ABI PRISM 7000 Sequence Detection System (Applied Biosystems, ABI). DNA was amplified in volumes of 4 µl, which corresponds to the amount of DNA present in 32 µl of maternal plasma. For a single copy sequence targeting assay, this allows for a theoretical detection limit of 31 GE per ml. Reactions of 25 µl contained 300 nM of each primer (HPLC purified, Microsynth, Switzerland), 200 nM of the MGB probe (ABI), and a 1 x concentration of the TaqMan Universal PCR master mix containing Amperase UNG (ABI). Primer and probe sequences were as follows: SRY-specific F: TCC TCA AAA GAA ACC GTG CAT, R: AGA TTA ATG GTT GCT AAG GAC TGG AT and probe TCC CCA CAA CCT CTT; DYS14-specific F: GCC AAT GTT GTA TCC TTC TC, R: CCC ATC GGT CAC TTA CAC TTC and probe: TCT AGT GGA GAG GTG CTC. The probes were MGB modified and labeled with FAM (SRY) and VIC (DYS14).

The reactions were prepared on ice. Cycling conditions were 2 min at 50 °C and 10 min at 95 °C for initial denaturation of the DNA and polymerase activation, followed by 50 cycles of 1 min at 60 °C and 15 s at 95 °C. The analysis was performed by the ABI Prism 7000 SDS Software with automatic baseline setting.

For the quantification of DNA from plasma samples we used an approach similar to the importing of standard curves (2): We determined the average C_T ($C_{T, \text{experiment}}$) of a single reference sample of known concentration (540 and 54 GE per PCR for SRY and DYS14, respectively). This reference sample was amplified in triplicate in each experiment. We calculated the theoretical C_T ($C_{T, \text{cal}}$) for the copy number in the

reference sample by the equation of the calibration curve. To use the standard curves generated in the assay validation, a correction factor ($C_{T,cor}$) was calculated: ($C_{T,cor} = C_{T,experiment} - C_{T,cal}$). The Intercept of the equation of the calibration curve was corrected by this $C_{T,cor}$ to receive the standard curve used for the experiment.

As replicates with no amplification are not included in the calculations of the SDS software, we quantified individual replicates and determined mean, standard deviation (SD) and coefficients of variation ($CoV = SD / \text{mean}$) from these quantities.

For sex determination with the DYS14 protocol we used a cut-off of 1 GE per PCR to discriminate between true positives (male) and unspecific amplification (female). Reactions were performed in triplicates which all had to indicate the same sex for determination. With the SRY protocol sex was determined if all of the three replicates either showed amplification or not.

Results

In a validation experiment we amplified reference DNA at 5400, 540 and 54 GE / PCR in quadruplicates. Calibration curves were constructed by plotting the C_T as a function of the log of the template copy number. (SRY: $C_T = -3.335 \times \log(\text{copies}) + 39.22$; DYS14: $C_T = -3.388 \times \log(\text{copies}) + 36.31$). From these curves we determined the $C_{T,cal}$, which was 30.44 for DYS14 and 31.28 for SRY.

In the same experiment we analyzed twofold dilutions of template DNA between 5.4 and 0.08 GE per PCR with the DYS14 and SRY specific assays. The results demonstrate the increased sensitivity and reproducibility of the DYS14 protocol for low copy numbers. The quantitative results determined by the SRY assay deviate considerably between replicates, the CoV are greater than 50 %. It is noteworthy that many replicates without amplification occur at sample inputs below 5 copies. This may be due to amplification failure occurring at very low target amounts or simply because no target sequences are present in the reaction mixture. The DYS14 specific protocol allowed reproducible amplification of target amounts as low as 0.1 GE. The quantification of the low input samples is still precise and reasonably accurate, CoV range from 7 to 50 %.

Input copy number	5.4	2.7	1.36	0.68	0.34	0.17	0.08
copy number determined by DYS14	3.91	3.02	1.15	0.18	0.20	0.18	0.08
	3.32	1.78	1.09	1.04	0.18	0.18	0.20
	3.31	1.13	0.91	0.58	0.33	0.25	0.08
	3.28	1.36	0.96	0.97	0.16	0.15	0.16
averages	3.46 (7.6%)	1.82 (40.3%)	1.03 (9.5%)	0.69 (50.0%)	0.22 (30.0%)	0.19 (19.4%)	0.13 (39.1%)
copy number determined by SRY	0.14	1.97	3.41	0	0	0	0
	2.32	2.54	0	0.41	0	0	0
	2.70	3.35	0	0	0	0	0
	3.84	0	0	0	1.25	0	0
averages	2.25 (59.6%)	1.97 (62.9%)	0.85 (173%)	0.10 (173%)	0.31 (173%)	0	0
replicates w/o amplification	0 / 4	1 / 4	3 / 4	3 / 4	3 / 4	4 / 4	4 / 4

Table 1: 2-fold dilution series of reference gDNA, sample input determined by DYS14 and SRY-specific real-time PCR. Averages with CoV in parentheses. Quantities are in GE.

We compared both assays by analyzing 12 plasma samples of unknown fetal sex (gestational age between 11+6 and 13+5 weeks). Each plasma was extracted in

duplicate and each DNA analyzed individually. Invasive testing identified that 6 samples were from male and 6 from female pregnancies. The fetal sex of all 24 DNA extractions was correctly determined by DYS14 specific real-time PCR. With the SRY specific protocol we were able to determine 8 out of 12 male and 11 out of 12 female DNA samples, the other five did not allow determination. The amplifications with the SRY assay were very specific. Only two of 36 replicates from female pregnancies amplified with very high C_T values (corresponding to 0.8 and 1.8 GE). With the DYS14 protocol we observed 13 false amplifications even after extensive optimization of the protocol. However, the indicated quantities per PCR are all smaller than 0.6 GE which is 10 times less than all results from male pregnancies.

The measurements of the male pregnancies are very precise with the DYS14 protocol (figure 1). The CoV range from 4 to 22 % in 12 triplicate measurements. None of the total 36 reactions failed with fetal DNA quantities determined between 5.7 and 19.4 GE per reaction. The SRY protocol resulted in much greater deviations (CoV between 26 and 140 %) and 6 negative replicates. For duplicate extractions, the average CoV were 16.9 % (13.8 – 27.8 %) for DYS14 and 74.2 % (63 – 103 %) for SRY. The CoV of the duplicate extractions together indicate that the SRY protocol is not more than semi-quantitative. Surprisingly the DNA levels determined by the DYS14 protocol were 2.5-fold higher than by SRY (range 1.6 – 4.3 fold).

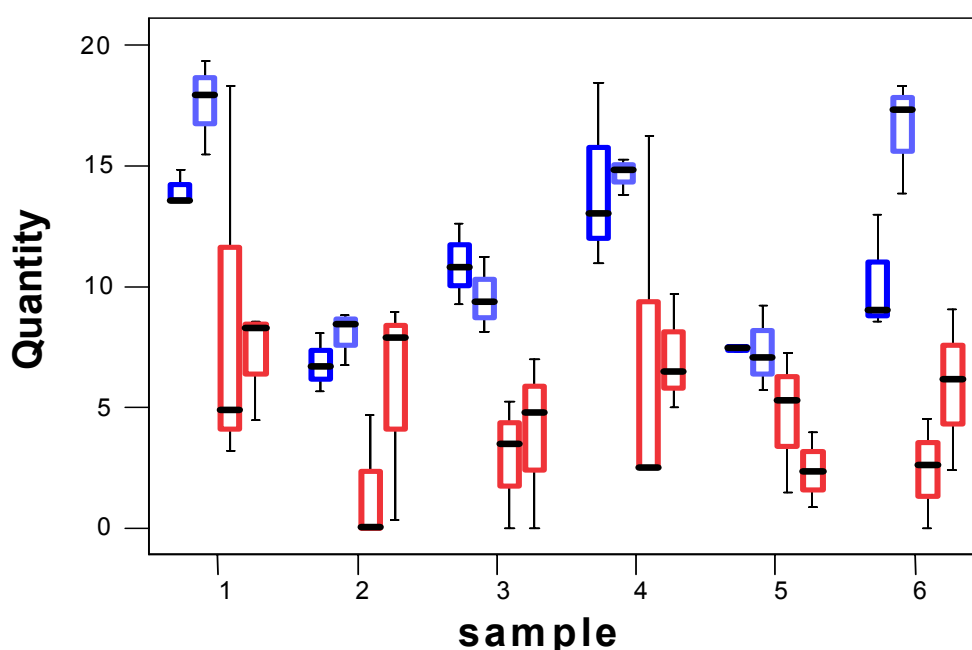
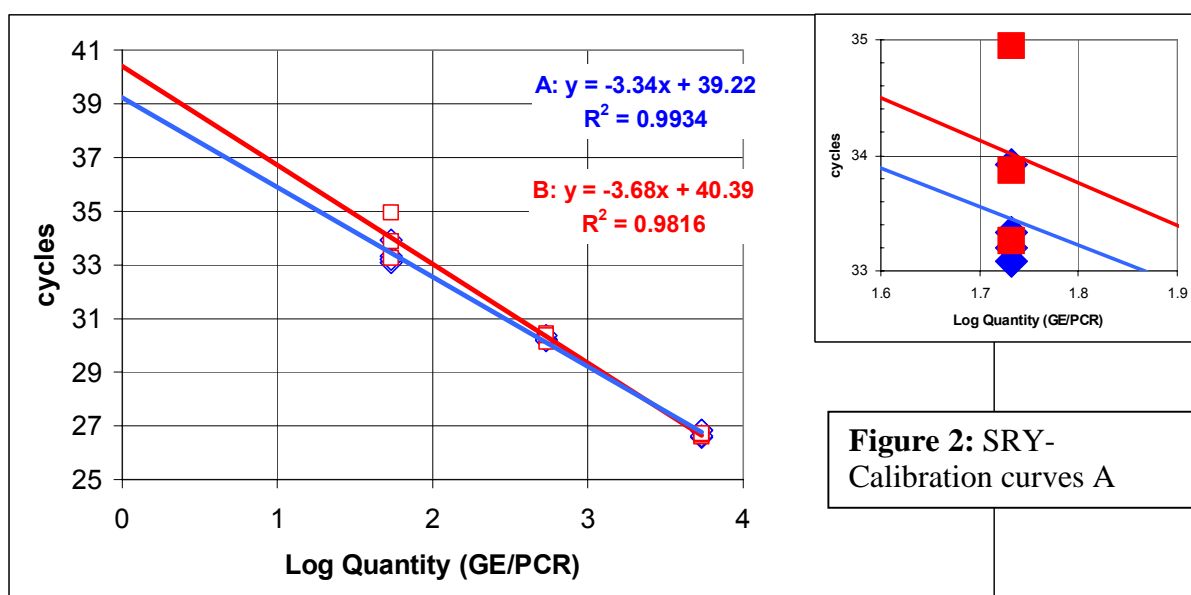


Figure 1. Duplicate extractions of samples 1 to 6 quantified by DYS14 (blue) and SRY (red).

To investigate the discrepancy between the copy numbers determined by both assays, we amplified genomic DNA dilutions in triplicate with DYS14 and SRY. With these amplifications we constructed new calibration curves and quantified 4 male plasma extracts. In three samples both assays determined the same copy number, while in one sample the SRY-result was three times lower (3.7 versus 11.2 GE per PCR). The old and new standard curves generated for the DYS14 protocol were very similar. In contrast the SRY curves. We plotted the old standard curve A, which we used for the quantification by a single reference sample, and the fresh curve B (figure 2). While the average C_T of the reference samples with copy numbers of 5400 and 540 GE are within 0.1 cycles, the curves diverge due to the differences of the 54 GE standard. The differences in the C_T of this concentration result in a difference of more than one cycle at the intercept. Using B instead of A for the imported standard curve would result in two-fold greater template numbers in reactions with less than 10 GE. This would lead to similar determinations by SRY and DYS14 in the plasma samples. However, the R^2 for A is much better than for B. The problem is, that the determinations of the lowest standard are too imprecise to allow correct quantification. The deviations increase for measurements of lower copy numbers, which will be skewed by random variability. The implication is that copy numbers for the plasma samples as provided by the SRY protocol can only be considered a rough estimate. The copy numbers we determine by the SRY assay could be just as well twice their value. By using new standard curves with every run, the inter-assay comparability of results would be reduced and no



accuracy gained. Standards of adequate concentration have similar copy numbers as the samples. However, with the SRY protocol neither can be measured accurately.

We further tested the specificity of the DYS14 protocol on 34 plasma samples at a gestational age of 11+6 to 13+6 weeks in a blinded manner. Again the sex of all samples was correctly determined.

Overall, 25 male plasma DNA samples were quantified between 3.4 and 31.5 GE per PCR with CoV between 7 and 27 %. In the 33 extractions of female pregnancies we observed amplification curves in 36 of 99 reactions. All these were indicative for less than 0.9 GE per PCR. As we applied a cut-off of 1.0 GE for the sex determination, none of the female replicates was assessed to be male.

We achieved a specificity and sensitivity of 100% for sex determination by the novel DYS14 specific assay in 58 DNA samples from plasma (46 different pregnancies). In the 17 male pregnancies of normal outcome, with gestational ages from 11+6 to 13+6 weeks, the average fetal DNA content was 426 GE per ml plasma (108 – 983). In the sample cohort, 2 pregnancies were trisomy 21 affected, their fetal DNA levels were 333 and 750 GE at 13 and 12 weeks of pregnancy.

The quantitative and qualitative limits of the novel assay are both around 1 GE per reaction, which is a clear advancement in comparison to the assay targeting the single copy gene SRY.

Discussion

The measurement of circulatory fetal DNA has been shown to correlate with certain pregnancy associated disorders (3). It is to be expected that in the near future large studies will be performed to assess the suitability of cffDNA as a marker for first and second trimester screening. The measurement of male fetal DNA levels is considered to be a simple procedure. However, as we could observe in a recent comparison study between five laboratories, quantitative data collected can differ significantly between sites (4). Even the use of the identical sample material and implementation of the same DNA extraction and real-time quantitative PCR procedures resulted in major differences of the finalized data. In the comparison of numerical values from different studies with varying protocols, major discrepancies can be expected. This lead to the pessimistic suggestion that absolute fetal DNA levels as determined by different sites might not be comparable even with a highly standardized protocol.

We show that when using a single copy gene locus as amplification target, the determination of fetal circulatory DNA levels of less than 10 GE per PCR cannot be considered quantitative. CoV from such reactions can reach over 100 %, which evidently does not allow a credible determination of two-fold differences. Our judgment is supported by the recent reporting of reduced reproducibility in samples with 10 copies and inconsistent detection and loss of linearity in samples with single copies (5).

However, a review of the literature has revealed that template numbers from reports of increased cffDNA levels in pregnancies with trisomy 21 fetuses as well as those disputing this finding are in the range of reduced quantitativity (6) (table 2).(7-10) In all these studies, no mention of precision of the copy number determinations is included. However, judging from our evaluation, the single measurements contain a high degree of uncertainty, and the data from these studies at most allow to observe trends for elevation. To determine if the cffDNA level of an individual sample can be used for the assessment of the risk for trisomy, quantification needs to be precise and strategies need to be optimized. To perform evaluation of cffDNA levels as a screening marker from such data seems premature and will probably underestimate the suitability of the approach (11). In addition, in many reports measuring plasma DNA levels it is difficult to determine the exact detection limit of the PCR in GE per milliliter plasma from the abbreviated protocols. This shortcoming makes the evaluation of results cumbersome if not impossible.

Author	extracted Plasma	elution volume	DNA per PCR	Plasma per PCR	Detection Limit (GE/ml plasma)	median GE/ml plasma controls	median GE/ml plasma trisomy 21	gest age
Lo	400 - <u>800</u>	50	5 *	80	12.5	16.3 (n=18)	48.2 (n=6)	17.2 / 18.0
Zhong	400	50	2	16	62.5	83.1 ^a (n=29)	185.6 ^a (n=15)	14+4
Lee	400	100 **	<u>5-10</u> **	40	25	~10-30 [§] (n=55)	~15-25 [§] (n=11)	15-19
Hromadnikov	400	50	2.5	20	50	24.5 (n=13)	23.3 (n=11)	15-22
Spencer	400	50	5	40	25	34.1 (n=10)	32.0 (n=10)	15

Table 2. Literature of extraction protocols, real-time PCR detection limits and median cffDNA levels reported in control and trisomy 21 pregnancies. Underlined numbers are used for our calculations. Volumes are in microliter. ^a: mean. * as reported in: Lo et al, Clin. Chem 1999;45:184-8; ** "similar to" Bianchi et al, Clin Chem 2001;47:1867-9; [§] as determined from figures.

More than 10 target sequences need to be present per reaction to generate sound data. This can be accomplished by increasing either the volume of plasma extracted or the copy number of target sequences per genome. Consequently, reference reactions for quantification should contain more than 10 target sequences and variability in replicate amplifications should be minimal. At the same time, copy numbers should be in the range of the samples to be quantified.

When we compared the plasma DNA levels determined by both assays, we made the surprising observation that levels determined by the DYS14 protocol are generally two times greater than levels determined by SRY. These discrepancies are a consequence of the great variability of the SRY based measurements. This finding revealed another flaw of the single copy SRY protocol: As the number of template sequences per reaction is very low, several factors can lead to less efficient amplification and a resulting underestimation of the copy number: amplification failure and a lag in the initial cycles and increased variability will result in the determination of lower amounts than actually in the reaction (5). Especially the relatively high background of maternal DNA and formation of primer dimers can generate a decreased efficiency or even amplification failure (12). These problems are effectuated by PCR competition from amplifications of unspecific artefacts.

Moreover, the levels determined in our study are also markedly higher as compared to those in the literature. This is partially due to the low amount of template affecting the enumeration. Additionally, since all samples were from patients with an increased risk for chromosomal aberrations as determined by first trimester prenatal screening, our cohort might have higher levels than in inconspicuous pregnancies. Importantly, a more efficient DNA elution may have lead to higher DNA recovery, as we took care to solubilize the DNA under heated conditions prior to elution from the filter (4). Consequently, for quantitative studies it will be important to monitor the DNA recovery. Additionally, we aimed to reduce loss of fetal DNA in the extracted solutions to a minimum and avoided freezing the extracts (13).

Finally, quantitative differences can arise from the absolute quantification by real-time PCR: Standard samples used can introduce discrepancies by differing determination of concentration, presence of inhibitors, use of different concentrations for the standard curve or reference sample. Also the slopes of standard curves can largely be affected by amplification variability.

When we performed a BLAST search with the primer and probe sequences from the original DYS14 targeting protocol, we observed considerable homologies to sequences on chromosomes other than Y (14); (15), which would lead to unspecific amplification from the maternal background DNA. This is probably the reason why the assay has only been applied to samples of confirmed pregnancy with a male fetus. However, unspecific amplifications at high rates might even affect quantification in extreme cases, for example when the background of maternal DNA is very high. It proved to be very difficult to develop an assay without any unspecific amplification, as homologies to sequences on other chromosomes could not be avoided fully. Although the assay was not designed for the determination of fetal sex, the susceptibility of the assay for unspecific amplification can be overcome by selecting a cut-off at 1.0 GE per PCR. By this approach we were able to exclude false male interpretations, as we showed in our blind analysis of totally 58 plasma extractions. Fetal sex determination may be possible at even earlier stages of gestation than reported with single copy locus specific assays (Rijnders 2001).

Here we present an approach to address one of the major hindrances in the generation of precise and relevant data, the scarce nature of the material to be quantified. We describe an assay with a ten times increased detection and quantitative limit as compared to the single copy sequence targetting protocol. This approach will be

useful in large studies for the assessment of cffDNA levels as possible marker of pregnancy associated disorders.

In the context of clinical application it will be important to standardize all procedural steps that lead to variability of the quantitative data. For large studies, thoroughly evaluated and optimized protocols need to be established. With a detailed description and discussion of procedures we try to highlight several important points to generate accurate data. Results should be reported in more detail. It is essential that in addition to the results in copy number and their CoV, detection and quantification limits should be included, as only these data allow assessment of the validity of the results. Further statistical analysis of the data only makes sense when one is aware of it's quantitative value.

In conclusion, the measurement of multicopy Y-specific sequences by real-time quantitative PCR is an optimal tool for the assessment of male fetal DNA concentration in maternal plasma. With the assay here presented the investigation of elevated cffDNA levels under a variety of pregnancy-related pathologies can be conducted in a quantitative manner.

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Inability to Reproduce Increased Percentage of Fetal DNA from Maternal Plasma.

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Chapter 11

To the Editor of the Journal of the American Medical Association:

The discovery of cell free fetal DNA in maternal plasma opened a new avenue for the risk free assessment of fetal genetic traits ¹. As the detection of fetal genetic loci completely absent from the maternal genome has proven to be highly specific, reproducible and quite facile, this method is already being used clinically for the determination of the fetal Rhesus status ².

The analysis of other fetal genetic loci not completely disparate from the mother has, however, proven to be very problematic, as their detection is masked by the vast abundance of maternal DNA sequences ². In this context, Dr. Dhallan and colleagues recently described a procedure whereby maternal blood samples were treated with formaldehyde ³. This was suggested to stabilize maternal blood cells, preventing the release of their DNA, thereby significantly enhancing the proportion of fetal DNA in maternal plasma samples. It was also suggested that this could form the basis for future investigations examining complex fetal traits, such as aneuploidies or single gene disorders.

Intrigued by this finding we have attempted to verify it. In our examination of 21 maternal blood samples, we performed a side-by-side analysis of plasma prepared by our standard protocol, (1600 x g for 10' at 4°C & 16,000 x g for 10' at RT) and by the formaldehyde method ³. The concentration of cell free maternal DNA was then determined by real-time PCR in each of these paired plasma samples. This analysis indicated that the concentration of maternal DNA was not significantly ($p= 0.32$) reduced by formaldehyde treatment (mean = 4286 copies/ ml plasma) when compared to conventionally prepared samples (mean = 4737 copies/ ml plasma).

It is, therefore, highly unlikely that the method proposed by Dhallan and colleagues ³ will lead to a preferential change in the ratio of fetal to maternal DNA. Instead, it appears that a misinterpretation of the data may have occurred by the use of an inappropriate PCR assay. In this regard, this report serves to highlight a recent finding made during the large scale NICHD "NIFTY" study, in that considerable care should be exercised when performing quantitative analysis of cell free DNA⁴.

Other strategies will, hence, have to be explored for the analysis of complex fetal genetic traits, such as the use of size-fractionated plasma DNA, whereby fetal sequences are preferentially enriched for on the basis of their smaller size than maternal ones ⁵.

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Nucleic Acid Based Biosensors: the Desires of the User.

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Chapter 12

Written for Biochemistry

ABSTRACT

The need for nucleic acid based diagnostic tests has increased enormously in the last few years. On the one hand this has been stimulated by the discovery of new hereditary genetic disease loci following the completion of the Human Genome Project, but also by the presence of new rapidly spreading viral threats, such as that of the SARS epidemic, or even micro-organisms released for the purpose of biological warfare. As in many instances rapid diagnoses of specific target genetic loci is required, new strategies have to be developed which will allow this to be achieved directly at the point-of-care setting. One of these avenues being explored is that of biosensors. In this review, we provide an overview of the current state of the art concerning the high-throughput analysis of nucleic acids, and address future requirements, which will hopefully be met by new biosensor-based developments.

Key words: DNA biosensor diagnosis PCR microarray

INTRODUCTION

Probably no event has altered clinical analysis of DNA and RNA such as the development of PCR (polymerase chain reaction) (1). The introduction of this technology permitted a examination of small quantities of material, even as little as a few or single cells, with the result being obtained within hours. This was in stark contrast to methods used till then, such as Southern (for DNA) or Northern (for mRNA) blot analysis which relied on microgram quantities of pure nucleic acids, and where a result was usually obtained only after a few days or even weeks.

It is therefore little wonder that PCR was readily seized upon by diagnostic or clinical laboratories, and that these in turn introduced many of the “cutting edge” applications, which later found widespread use in the bio-medical research community. Examples of this are single cell PCR (SC-PCR), which was pioneered for pre-implantation genetic diagnosis (PGD) (2), and allele-specific PCR, which permitted the rapid determination of mutant loci, which frequently differ by only a single base change from the normal allele.

The elucidation of genetic loci implicated in hereditary genetic disorders (e.g. cystic fibrosis), or those which were associated with a risk for a particular disease later in life, (e.g., the BRCA1 and 2 genes and the development of breast cancer), has driven the development of new PCR-based technologies which permit a high-throughput analysis of the numerous mutant alleles associated with such disorders. Of these technologies, probably the most promising is the use of high density nucleic acid arrays (3), also termed DNA Chip Technology, which permits the simultaneous assessment of numerous genetic alleles or loci.

A further technological development of the PCR process, which has found widespread clinical applicability, was the advent of real-time PCR, which facilitated the highly accurate quantitation of nucleic acids (4). This technology has rapidly been applied to clinical settings, especially in oncology, where it is used for the determination of gene dosage or the associated level of gene expression of genes implicated in the malignancy. It has, however, also been widely used in the examination of infectious diseases, for instance in monitoring the viral burden in HIV infected individuals undergoing a variety of treatment regimens, to assess drug efficacy.

Although the development of PCR, in all its various forms, has completely altered the analysis of nucleic acids for diagnostic purposes, it is to be expected that the next

technological revolution will equally transform applications as we know them today. It is to be expected that this new revolution will again be driven by the requirements of the end-user, which amongst others are:

- cheap, rapid, simple,
- high-throughput
- ability to be used directly at the point-of-care setting by staff with no laboratory training.

This latter point is gaining in importance as many clinical or other analytical applications increasingly rely on near-instantaneous answers directly at the point-of-care or point-of-entry setting. Examples of these include:

- The rapid identification of infected individuals or carriers for a new viral epidemic e.g., SARS, directly at the point-of-entry into a country.
- Rapid assessment of the fetal genotype in prenatal diagnosis.
- Rapid assessment of the oncological status of a malignant lesion directly in the surgery.
- Viral load response to drug treatment.
- Detection of genetically modified organisms or foodstuffs.
- Detection of bacterially contaminants during food processing.
- Detection of biological agents used for germ warfare.

These facets will permit much more rapid therapeutic or interventional strategies leading to better and more cost-effective healthcare management.

These few scenarios do, however, imply that this next phase cannot be addressed by current technologies, and that new tools will have to be developed which are as simple to use as current technologies permitting diabetics to monitor their own blood sugar levels in a minimally invasive manner. In as much as such glucose monitoring devices rely on biosensor technologies, it is hoped that the new revolution of nucleic acid analysis will similarly be introduced by novel DNA-based biosensors.

CURRENT STATE OF THE ART OF HIGH DENSITY DNA ARRAYS

Microarrays are miniaturised analytical systems emerging from traditional biochemical assays such as Southern, Northern or Western blotting (5, 6). According to these conventional assays different branches of microarrays evolved which can be

applied in a complementary fashion to achieve a comprehensive global view of genes and gene expression, posttranslational modifications and protein status. The miniaturisation of the assay systems enables the simultaneous interrogation of either many different events within one biological sample e.g. a specific cell population under certain stimuli, or the testing of a multitude of samples in parallel for the same event. Both variants provide a high throughput analysis tool which can be adapted to most different types of experimental questions and customer needs.

Application fields for micro-arrays include diagnostics with genotyping applied to either mutation detection in specific human disease-related genes or identification processes in microbiological tasks, or gene profiling for predictive medicine. In the latter scenario, transcription patterns are correlated with different pathophysiological conditions, prognoses and disease progressions. Consequently the technology can be used for superior patient-tailored treatment based upon recognition of specific aberrant signalling pathways and expression patterns. A second field encompasses the identification of novel biomarkers and new targets for drug therapeutics as well as monitoring their effects e.g., efficacy and toxicity, on the organism.

The fundamental basis of the microarray technology is a process of hybridisation where two biological molecule pair together in dependence of their complementary sequences or structures: DNA/DNA, cDNA/RNA, protein/antibody. The application of more novel interactions, such as those with aptamers, affibodies or glycoproteins for microarray applications is currently being explored.

Technically advanced robots permit the disposition of probe molecules (oligonucleotides, PCR-amplified cDNA-fragments, proteins, antibodies) in micrometer distances onto the array surface, thereby facilitating the immobilisation of many different probes in a high density array on small surfaces of e.g. one square centimeter. The oligonucleotide probes can either be synthesised step by step in silico using photolithographie (allowing for a density of 10^6 different oligonucleotides per cm^2) or they are pre-synthesised in vitro and subsequently spotted onto the array surface (10000 – 30000 spots per slide); this surface usually consists of nylon membranes or glass slides coated with various agents providing immobilisation while maintaining probe native structures. The generation of oligonucleotide or cDNA arrays requires highly sophisticated bio-informatics systems as care needs to be taken that the maximum hybridisation-potential for every single array-probe-sample is achieved.

For the analysis of gene-expression, also termed gene-profiling, the sample RNA is generally converted into stable cDNA, using approximately 5-10 ug total RNA as starting material. Amplification of this sample material is usually achieved by in-vitro transcription or PCR. During this procedure the sample is also labelled with a fluorophore used for the identification of target-probe interactions in the subsequent hybridisation process. Quantitative analysis can either be obtained using a single colour scheme, where the fluorescently labelled sample is hybridised to different gene arrays, or by the use of multi-colour settings, whereby two samples which are to be compared are labelled with different fluorophores and co-hybridised to the same array.

As these high-density arrays permit the simultaneous examination of several thousand loci, new bio-informatic tools have had to be developed in order to facilitate useful data mining, which by careful correlation with the underlying physiological processes will lead to the extraction of biologically important information from this wealth of data.

Although a major application to date of such high density arrays has been for the purpose of gene profiling, great strides have recently been made in applying this technology for genotyping or disease loci or of single nucleotide polymorphisms in order to determine disease associated patterns (7).

CURRENT STATE OF THE ART OF REAL-TIME PCR

Traditionally, PCR was a qualitative method, that was rendered semi-quantitative by ingenious reaction set-ups, such as competitive PCR where standards of known amount are co-amplified with the sample or quantitative fluorescent PCR.

This deficit has been completely changed by the introduction of real-time PCR analysis (4), which has revolutionized the quantitative analysis of DNA and RNA. In real-time PCR reactions the concentration of the input template nucleic acid is accurately determined by the accumulated emission of a fluorescent signal during each cycle of the PCR reaction. This system permits the accurate quantitation of sample concentration over a dynamic range of several orders of magnitude. Furthermore, the system has proven to be very reliable and sensitive, being capable of detecting small amounts of target in the order of 1 to 10 copies. It is suitable for the determination of small differences in copy numbers, as witnessed from our recent

description of an assay which permits the detection of fetal aneuploidies (8), where only a 50% increase in target template occurs.

A significant advantage of real-time PCR is that no post PCR handling is required, such as gel electrophoretic analysis of the PCR products, which effectively eliminates the risk of carry-over contamination, a vitally important feature for clinical settings.

As the assay can be accessed in real-time, several quality controls can be integrated to monitor the efficacy of the PCR assay, such as final fluorescence, replicate uniformity, slope of the fluorescence curve and melting curve analysis.

Furthermore, full advantage can be made of the fluorescent signal detection system, in that currently up to 4 different fluorophores (including a passive loading control) can be monitored simultaneously. In addition the system is very amenable to automation and is constantly being adapted to new formats which permit high-throughput, such as the recently developed 384 well format, which requires very little reagent chemistry (as little as 5-20 μ l) and permits a very rapid PCR cycles. This implies that by the use of 4 different fluorophores, 1536 different targets can be simultaneously analysed in a real-time setting, with a result being obtained in as little as 10 minutes.

It is hence, no wonder that since its introduction a few years ago that real-time PCR has been used for a myriad of applications including, gene profiling, mutation and SNP analysis, monitoring of viral load, to the analysis of cell free DNA concentrations in a variety of disorders (9-10).

It is, hence, to be expected that real-time PCR will continue to spearhead developments concerning the rapid and highly precise analysis of nucleic acids.

BIOSENSORS: WHERE DO WE STAND FROM A USERS POINT OF VIEW?

It readily becomes apparent from a quick perusal of the current literature that numerous DNA-based biosensor systems are being evaluated, and that new formats are almost being added on a daily basis. As these are being addressed in much greater depth in other contributions to this volume, we shall only briefly describe a few here and elude to current constraints limiting their immediate or near future use in a clinical or diagnostic setting.

In general the underlying physical properties of the various types of sensors use the following modes of detection: fluorescence, electrochemical or altered surface properties.

One of the most promising approaches for the fluorescent detection of nucleic acids appears to be the use of fibre optic systems, where in the interim it has become possible to coat pits chemically etched into individual optic fibres with a single sequence specific probe, thereby in essence permitting the analysis of single molecule – target sequence interactions (11). A highly sensitive CCD camera is used to monitor sequence specific hybridisation on the fibre bundle, which have been arranged into block-matrixes, to facilitate large scale parallel analysis. By using such an approach it has recently been possible to profile transcript differences in a panel of human cancer cell lines, using starting material from as few as 10 cells.

Electrochemical applications are also being intensively explored, as these are potentially cheaper to operate and maintain than the more complex systems required for the analysis of fluorescent signals. In these approaches both the analysis of nanoparticles as well as solid phase array biochips is being explored.

In the former approach, DNA oligonucleotides are spotted in arrays which are flanked by electrodes (12). Following hybridisation of the target sequence, the combined probe-target sequence is then recognised by gold nanoparticles coupled to specific oligonucleotides, which is used to trigger an electrical signal in combination with a reactive silver nitrate solution. By using this approach highly specific sequence detection with femtomolar sensitivity were reported.

In parallel developments electrical biochip technologies are also being explored (13). In these systems, ELISA-based enzymatic redox reactions, such as the conversion of the substrate p-aminophenyl phosphate to the electroactive p-aminophenyl by alkaline phosphatase, are used to register the binding of specific target molecules. Potential applications of this technology which are under investigation include the monitoring of viral infections. Advantages of electrical biochips are that this technology is readily adaptable for a variety of biological targets, such as nucleic acids, metabolites (glucose) or proteins (antigens). Furthermore these devices are highly sensitive, do not require mechanical adjustments, are simple to use and readily miniaturised.

Other research avenues include the use of quantum dots (14) and nanomechanical cantilever arrays (15, 16). Quantum dots are generated from discretely sized nanocrystals of cadmium selenide capped with zinc sulfide (14). The advantage of these dots is that they have unique optical properties, such as the ability to emit single colour signals at different wavelengths depending on the size of the dot. Furthermore, even if the dots are close proximity of each other no FRET (fluorescence resonance

energy transfer) effect occurs. This implies that highly dense arrays in the nano or micrometer scale are possible. The advantage of these dots is that as they very small, they can be incorporated in a vast array of combinations into polymer microbeads. By labelling each of these microbeads with a sequence specific oligonucleotide, this system permits rapid and efficient gene profiling.

Nanomechanical cantilever arrays, on the other hand, make use of physical changes in surface structure, such as the bending of a micro-cantilever upon the specific binding of a target molecule (15, 16). The sensitivity of this system can be significantly improved by the inclusion of a magnetic control system. In this manner the deflection of the cantilever is enhanced by the application of an external magnetic field which acts on target sequences bound to the cantilever, which have been tagged with oligonucleotides labelled with magnetic particles.

Although these approaches are all very promising, their current sensitivity is in the nanomolar range. Furthermore, most experiments have been restricted to the detection of artificial oligonucleotide mixtures, and the specificity of these detection systems has not been tested under “real-life” conditions where the vast amount of nucleic acids do not correspond to the target sequence.

CONCLUSIONS

From this overview it should be clear that significant strides are not only being made with new biosensor technologies for the analysis of nucleic acids, but that considerable advances have been made with more conventional PCR-based approaches. This is evident from new developments in real-time PCR, where results can be obtained in a matter of a few minutes, or from DNA arrays where thousands of alleles can be examined simultaneously.

On the other hand, DNA sensor technologies, while promising, still need to bridge the gap between experimental status and the harder reality of clinical / diagnostic applications. Nevertheless, it is to be hoped that these technologies will lead to the development of new tools which are simple and cheap enough to be used at point-of-care sites, thereby addressing a real need which cannot be met by current PCR-based platforms.

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Outlook.

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Chapter 13

As most of my work involved the fascinating method of real-time quantitative PCR, I will also in the future address several projects involving prenatal diagnosis and PCR:

- A major focus will be to follow up on the real-time PCR method for the detection of aneuploidies. We will test the novel real-time PCR instrument GeneXpert ® of Cepheid for its suitability to perform the diagnosis. In combination with their cartridge-based automatic sample preparation system it could be feasible to perform a rapid test that gives a diagnostic result within minutes of the invasive sampling. This test could be performed at the point of care without the requirement for technical staff or further laboratory equipment .
- The concept to enrich the fetal fraction of the plasma DNA is one of the most promising approaches for non-invasive prenatal diagnosis. We will search for methods that permit a more straightforward enrichment with higher recoveries than the gel-based method. We will also try to develop novel methods for the prenatal detection of mutations, similar to the β thalassemia assays. With the implementation of real-time quantitative PCR or the more conventional quantitative fluorescent PCR even aneuploidy detection in a non-invasive manner is thinkable.
- Real-time quantitative PCR is a method of tremendous potential. The detection and quantification of low copy numbers and in multiplexed amplifications require optimal reaction conditions and analysis. In this respect I will further investigate the phenomenon of PCR bias, which I discovered during my large trisomies study. I will also search for reaction conditions that allow more specific, highly sensitive and cheaper detection of sequences. Similarly, new concepts to thoroughly evaluate and compare different reaction conditions need to be established.
- We already started a large study for the evaluation of fetal DNA levels in maternal plasma with the novel multicopy-sequence targetting DYS14 assay. In this work we will also aim to further monitor and standardise DNA recovery and quantification.
- The most recent candidate as a marker for pregnancy-related disorders is particle associated mRNA of placental origin in the maternal circulation. We will establish

experimental procedures for the measurement of promising gene-transcripts, and evaluate their usefulness for prenatal screening.